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THE MECHANISM OF SULFONAMIDE RESISTANCE
IN EPISOMAL AND CHROMOSOMAL MUTANTS
OF ESCHERICHIA COLI

by



CRISTABEL A. STOCKTON

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled, " The Mechanism of Sulfonamide Resistance in Episomal and Chromosomal Mutants of Escherichia coli," submitted by Cristabel A. Stockton, in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The mechanism of resistance to sulfonamides was investigated in chromosomal and episomal mutants of Escherichia coli K-12. The study suggests that a number of factors may contribute to the apparent resistance.

Sulfonamides were shown to inhibit growth of the sensitive parent strain, Escherichia coli K-12, by a factor of 500 times more effectively than they inhibited either the chromosomal or episomal mutants. In the synthesis of folate compounds by cell-free systems, however, sensitive and resistant strains were equally sensitive to the action of sulfonamides. Kinetic studies, using dialysed ammonium sulfate enzyme fractions from sensitive and resistant strains of E. coli K-12-AB-301, were inconclusive. There was no apparent difference in the K_m values for p-aminobenzoic acid between sensitive and resistant strains.

The specific activity of the folate-synthesizing enzyme(s) from resistant strains was twice that of the enzyme(s) from the sensitive strain.

In both episomal and chromosomal sulfonamide-resistant mutants of E. coli K-12-AB-301, the amount of folic acid synthesized in vivo was approximately 2-fold greater than that synthesized by the sensitive parent strain. In the presence of subinhibitory concentrations of sulfadiazine during growth, folate synthesis was reduced by one-half in both sensitive and

resistant strains. The total folate levels in the resistant strains were still two times greater than that of the sensitive strain.

Intracellular and extracellular ρ -aminobenzoic acid pool levels were measured. The episomal and chromosomal mutants both appeared to have pool levels 1.5 to 2.0 times greater than did the sensitive strain.

Uptake studies using ^{35}S -sulfadiazine were inconclusive. Difficulties encountered with the uptake procedure made it impossible to demonstrate sulfadiazine uptake in any system.

^{14}C - ρ -aminobenzoic acid uptake was shown to occur in sensitive and resistant strains. In the presence of sub-inhibitory concentrations of sulfonamide, ρ -AB uptake was decreased in both episomal and chromosomal mutants but not in the sensitive strain, E. coli K-12-AB-301.

On the basis of the foregoing results, no one mechanism appears to be responsible for the resistance to sulfonamides. Several factors - folate levels, enzyme activities, ρ -aminobenzoic acid levels, inhibition indices and possibly permeability - may all contribute to the overall development of sulfonamide resistance.

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ABBREVIATIONS

AMP	- adenosine monophosphate
ATP	- adenosine triphosphate
ACG	- yeast extract-casitone-glucose media.
ρ AB	- ρ -aminobenzoic acid
ρ ABG	- ρ -aminobenzoylglutamic acid
biot	- biotin
CM	- chromosomal mutant
DHP	- dihydropteridine
DHPte	- dihydropteroate
DHF	- dihydrofolate
EM	- episomal mutant
H_2 -pteridine- CH_2OH	- hydroxymethyldihydropteridine
met	- methionine
ME	- mercaptoethanol
NTG	- N-methyl- N^1 -nitro-N-nitrosoguanidine
PP_i	- pyrophosphate
RTF	- resistance transfer factor
R factor	- resistance factor
S.A.	- specific activity

Sd	- sulfadiazine
Smer	- sulfamerazine
Smeth	- sulfamethazine
Sth	- sulfathiazole
THF	- tetrahydrofolic acid
Tris	- tris (hydroxymethyl) aminomethane
TM	- tris-maleic acid

INTRODUCTION

BIOSYNTHESIS OF FOLIC ACID AND RELATED COMPOUNDS

The biosynthesis of folate compounds from pteridine precursors and ρ -aminobenzoic acid was observed in whole cells of several bacterial species (Miller, 1944; Korte et al, 1957, 1958; Sevag and Ishii, 1958).

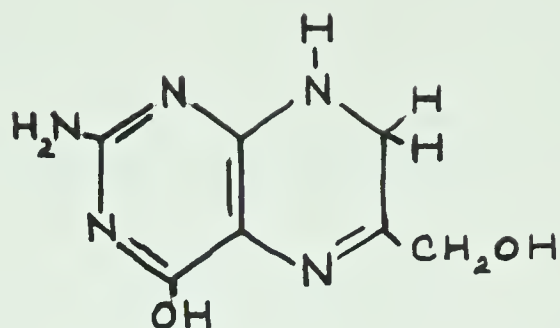
The first evidence of folate synthesis by bacterial cell-free extracts was obtained from preparations of Mycobacterium avium which, in the presence of xanthopterin (2-amino-4, 6-dihydroxypteridine), ρ -aminobenzoylglutamate (PABG), adenosine triphosphate (ATP), biotin and thiamine pyrophosphate, were able to synthesize folic acid (Katanuma and Shoda, 1958). Using 2-amino-4-hydroxy-6-hydroxy-methylpteridine as precursor, cell-free folate synthesis was demonstrated in Lactobacillus arabinosus 17-5 (Shiota, 1957) and in Escherichia coli (Brown, 1959).

The biosynthetic pathway for folate compounds has been elucidated (Shiota et al, 1961, 1962; Brown et al, 1961; Weisman and Brown, 1964; Richey and Brown, 1969; Shiota et al, 1969) as illustrated in Fig. 1.

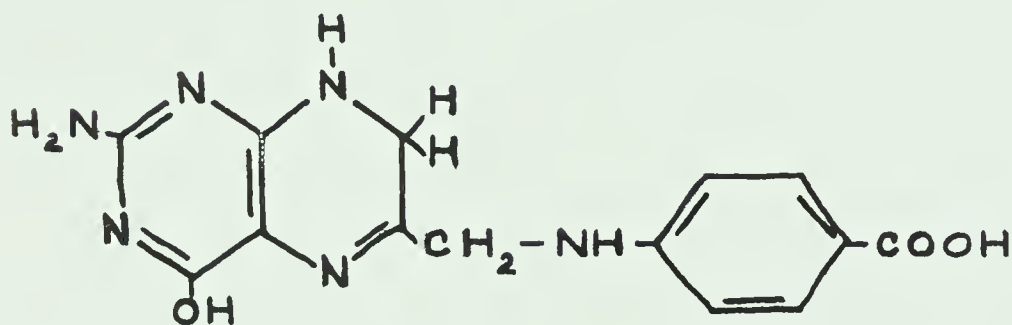
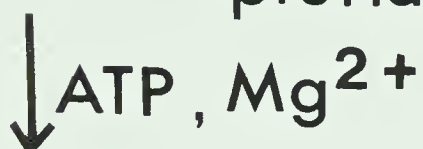
The initial studies on the enzymatic synthesis of folic acid carried out by Shiota (1959) and Brown (1959) showed that the only pteridines which were utilizable as substrates were either 2-amino-4-hydroxy-6-formylpteridine or 2-amino-4-hydroxy-6-hydroxymethyl pteridine or reduced forms



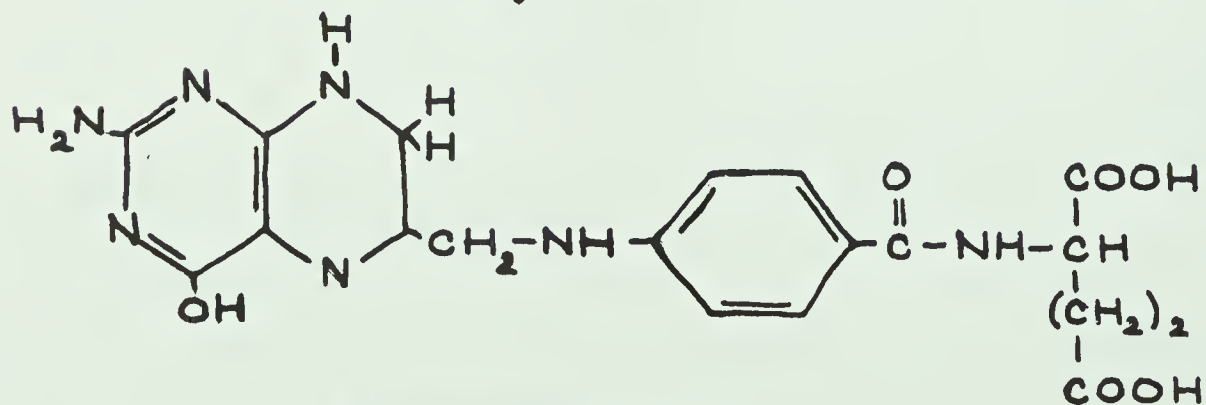
p-aminobenzoic acid



2-amino-4-hydroxy-6-hydroxymethyldihydro-pteridine



dihydropteroate



dihydrofolate

FIGURE 1

BIOSYNTHETIC PATHWAY OF FOLIC ACID

(in Brown et al., 1961)

of these compounds. Reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), in addition to ATP, were essential cofactors. It was later shown (Shiota and Disraeli, 1961; Brown et al, 1961; Wolf and Hotchkiss, 1963) that the true substrate of the reaction was the dihydro form of the pteridine; ATP was the only cofactor requirement in this case, suggesting that NADH and FAD were necessary only for the reduction of pteridines in the system.

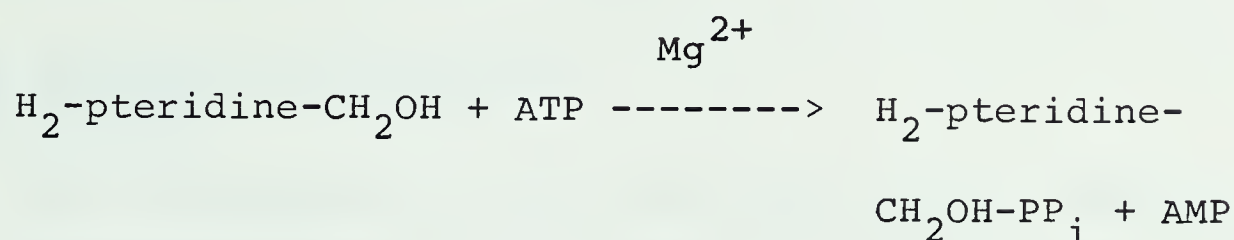
p-Aminobenzoic acid was shown to be an effective substrate for dihydropteroate synthesis in whole cells (Korte et al, 1957) and in cell-free systems (Shiota, 1959; Brown, 1959).

Substitution of p-aminobenzoic acid (pAB) results in a direct condensation reaction between the reduced pteridine and pABG to yield dihydrofolate and free pyrophosphate. Although Shiota (1959) observed no difference in the utilization of either pAB or pABG in the L. plantarum system, Brown et al (1961) reported that pAB was utilized for the enzymatic formation of dihydropteroate ten times more effectively than was pABG for the formation of dihydrofolate in E. coli. Further investigation has revealed that the purity of the enzyme preparation may affect the relative effectiveness with which the two substrates are utilized. Heating and fractionation procedures destroy the capacity of the E. coli system to utilize pABG. Weisman and Brown (1964) have suggested that two enzymes may be involved; however, the hypothesis that a single enzyme which can utilize both pAB and pABG but whose

activity toward ρ ABG may be destroyed by purification or handling cannot be overlooked. Results of fractionation of L. plantarum extracts by polyacrylamide gel electrophoresis have suggested that only one enzyme is involved, and that it is able to utilize either ρ AB or ρ ABG (Shiota et al, 1969). Further evidence that the folate biosynthetic pathway proceeds via ρ AB rather than ρ ABG has been obtained by isolation of an enzyme from E. coli that catalyzes the synthesis of dihydrofolate from dihydropteroate and glutamate in the presence of ATP, Mg^{2+} and monovalent cations (NH_4^+ , K^+ and Rb^+) (Griffin and Brown, 1964).

Demonstration of an ATP requirement for the coupling of hydroxymethyldihydropteridine to ρ AB to ρ ABG suggested that a phosphorylated intermediate might be involved in the pathway (Shiota, 1959; Brown et al, 1961). With extracts of L. plantarum and Veillonella, and in the absence of ATP only the pyrophosphate (PPi) ester of the pteridine was active in folate or pteroate formation (Shiota et al, 1962). The monophosphate ester could not be converted to pteroate or folate even in the presence of ATP. This suggested that pyrophosphorylation rather than step-wise phosphorylation must be an intermediate step in the folic acid pathway. These observations were confirmed by Weisman and Brown (1964) for the E. coli system and by Ortiz and Hotchkiss (1966) for Diplococcus pneumoniae. Richey and Brown (1969), using extracts of E. coli, and Shiota et al (1969) using L. plantarum have since isolated and characterized the enzymes required for the synthesis of

dihydropteroic acid. One of the enzymes, dihydrohydroxymethylpteridine pyrophosphokinase, catalyzes the pyrophosphorylation of the pteridine:



Isolation of the PP_i intermediate and identification of AMP have confirmed their results. The second enzyme, dihydropteroate synthetase, has been found to catalyze the synthesis of dihydropteroate from ρAB and the product of the pyrophosphokinase-catalyzed reaction. The reaction must involve a nucleophilic attack by the free amino group of ρAB or ρABG resulting in displacement of the pyrophosphate group. The release of PP_i has been confirmed by Richey and Brown (1969).

In the Pneumococcus system, Ortiz and Hotchkiss (1966) have reported inhibition of dihydropteroate synthesis by inorganic pyrophosphate. No such inhibition has been observed in either the E. coli or L. plantarum systems. There has been no evidence that a reversal of pteroate or folate synthesis may occur in any of the bacterial systems.

The magnesium ion requirement of the system has been confirmed for both the kinase and synthetase reactions (Weisman and Brown, 1964; Richey and Brown, 1969; Shiota et al, 1969; Ortiz. 1970). Enhancement of the synthetase activity by Mg^{2+} is in agreement with other enzymatic reactions that

involve displacement of pyrophosphate groups of nitrogen compounds. One example is the reaction involving synthesis of thiamine phosphate (Leder, 1961).

Natural Occurrence of Folates in Micro-organisms

The distribution of folic acid and related compounds in the microbial world has been confined mainly to the analysis of total folate products by conventional microbiological assay methods. Lactobacillus casei and Streptococcus faecalis have been used routinely for titrimetric and colorimetric analysis of total folates with little interference from other compounds (Tepley and Elvehjem, 1945). Differences in response are known to exist between the two organisms: (1) S. faecalis responds to pterioic acid while L. casei does not. (2) L. casei responds to methylated derivatives of folates but S. faecalis does not (Table I). A third organism, Pediococcus cerevisiae is of value in estimating nannogram quantities of 5 - and 10 - formyltetrahydrofolate derivatives (Sauberlich & Baumann, 1948).

Separation and identification of various naturally occurring folates was formerly achieved by a combination of paper chromatography and bioautography (Wieland et al, 1952). These methods have been replaced primarily by ion exchange chromatography techniques (Silverman et al, 1961; Sotobayashi et al, 1966).

Because most of the folate derivatives in micro-organisms exist in conjugated polyglutamyl forms which are

TABLE I

Response of Micro-Organisms to Various
Folate Derivatives

Compound	<u>S. faecalis</u>	<u>L. casei</u>	<u>P. cerevisiae</u>
Folate	+	+	-
Dihydrofolate (DHF)	+	+	-
Tetrahydrofolate (THF)	+	+	+
5-Formyl THF	+	+	+
10-Formylfolate	+	+	-
10-Formyl DHF	+	+	-
10-Formyl THF	+	+	+
5-Methyl THF	-	+	-
Folylglutamate	+	+	-
Folyl diglutamate	-	+	-
Folylhexaglutamate	-	-	-
THF diglutamate	?	+	+

From -

The Biochemistry of Folic Acid and Related Compounds. R. L. Blakley, North-Holland Publishing Co., London. 1969, p. 29.

generally inactive in microbiological assay systems, enzymatic hydrolysis with γ -glutamylcarboxypeptidase from chicken pancreas (Mims & Laskowski, 1945), hog kidney (Doctor & Couch, 1953) or pea cotyledons (Blakley, 1969) has been commonly carried out to release the bound forms.

Relatively few investigations have been carried out in bacterial systems to determine the kinds of naturally occurring folates that are present. Triglutamate forms of citrovorum factor (5- and 10- formyltetrahydrofolate) have been reported in S. faecalis and Bacillus subtilis (Zakrzewski and Nichol, 1955; Hakala and Welch, 1957); polyglutamyl pteridine derivatives have been found in Clostridium cylindrosporum (Wright, 1955). Studies with yeast cells have revealed significant levels of N¹⁰-formyl derivatives of folate, dihydrofolate or both, N⁵-formyl tetrahydrofolate (Schertel et al, 1965; Allfrey and King, 1950). p-Aminobenzoyl polyglutamates with 10-11 glutamic acid residues suggest that these forms may be highly conjugated.

Mode of Action of Sulfonamides

The use of sulfonamides as chemotherapeutic agents against certain species of the Enterobacteriaceae has been recognized for several decades; however, their biochemical mode of action was not fully understood until the folic acid biosynthetic pathway was elucidated. As early as 1940, Woods demonstrated that pAB was an antagonist of the bacteriostatic action of various sulfonamides. Other workers were able to show that sulfonamides inhibited the synthesis of folate compounds

in vivo in a number of different bacteria, and that the inhibition could be reversed competitively by ρ AB; on the other hand growth of folate auxotrophic bacteria was not affected (Lampen and Jones, 1946), 1947; Lascelles and Woods, 1952).

This suggested that sulfonamides must be competing with ρ AB for utilization as substrate in folate biosynthesis. This would not seem unreasonable in view of the similarity in chemical structure of the two compounds:



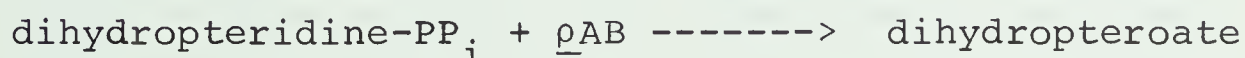
ρ -aminobenzoic acid



sulfonamide

In either compound the amino group remains free for the nucleophilic attack on the pyrophosphopterin complex.

Inhibition of the enzymatic synthesis of folate compounds by sulfonamides has been demonstrated (Brown, 1959, 1961). Several sulfonamides have been tested and results indicate that those compounds which were relatively effective inhibitors of the enzyme system were generally effective as growth inhibitors also. Kinetic studies in both E. coli and D. pneumoniae cell-free systems confirm a competitive inhibition by the sulfonamides of the condensation reaction (Brown, 1962; Wolf and Hotchkiss, 1963; Ortiz and Hotchkiss, 1966):



Labelling studies with ^{35}S -sulfanilic acid have indicated that sulfonamides may form a folate-like compound upon condensation with the pteridine in the presence of ATP and Mg^{2+} (Brown, 1962). No biological activity has been demonstrated for it. Hotchkiss and Evans (1960) have reported formation of a compound of silimar nature when *p*-aminosalicylate is added to the pneumococcus system; the compounds so formed showed folic acid activity for pneumococci but not for *S. faecalis*.

Mechanisms of Resistance to Sulfonamides

Microbial drug resistance was a problem of some concern that arose in the 1940's with the extensive use of chemotherapeutic agents for treating bacterial infections. The acquired resistance initially appeared in mutants of the sensitive strains whose genetic composition had been altered in some way; these were called chromosomal mutants and were obtained by step-wise exposure to increasing concentrations of sulfonamides (Oakberg and Luria, 1947).

Recognition of the anti-sulfonamide action of *p*-aminobenzoic acid and the role of sulfonamides in inhibiting the biosynthetic pathway has led to a series of investigations into the nature of the sulfonamide resistance mechanisms (Landy et al, 1943; Lascelles and Woods, 1952; Davis and Maas, 1952; White and Woods, 1965a,b; Brown, 1962; Pato and Brown, 1963; Ortiz and Hotchkiss, 1966, Ortiz, 1970; Kabins et al, 1971). Early studies proposed that chromosomally-resistant

strains of S. aureus were a result of increased levels of ρ AB capable of antagonizing the effects of sulfonamides (Landy et al, 1943; Oakberg and Luria, 1947). Analysis of cell filtrates indicated a 100-fold increase in ρ AB levels of resistant cells as compared to sensitive cells; this amount was far in excess of that required for reversal of sulfonamide inhibition. No such differences between sensitive and resistant strains have been reported for other bacterial species (Landy et al, 1943; Lascelles and Woods, 1952; Yaniv and Davis, 1953; White and Woods, 1965a).

In S. aureus it has also been found that there is an increased capacity of a resistant strain to synthesize folate from ρ AB as compared to the sensitive parent strain (Lascelles and Woods, 1952); the synthesis was also less sensitive to sulfonamide inhibition than was that of the parent strain. Sevag and Ishii (1958) have demonstrated a similar situation in E. coli. In cell-free systems no evidence for differences in the specific activity of the enzymes involved has been reported (Pato and Brown, 1963).

In 1952 Davis and Maas proposed a hypothesis that an enzyme altered in its affinity for ρ AB and sulfonamide was responsible for chromosomal drug resistance in E. coli. Their proposals had been based only on observations of growth inhibitory effects but when tested in the cell-free E. coli systems of Pato and Brown (1963) proved to be partially correct. A study of sulfonamide: ρ AB inhibition ratios in the cell-free system indicated that the enzyme from resistant cells had a

lowered affinity for sulfonamides. Kinetic studies on purified enzymes of the pneumococcus system have confirmed these observations (Wolf and Hotchkiss, 1963; Ortiz and Hotchkiss, 1966; Ortiz, 1970). No evidence of a higher affinity of the synthetase enzyme for ρ AB in resistant strains has been reported.

Decreased permeability of the cell membrane has also been considered an operative mechanism in some systems. Pato and Brown (1963) isolated a sulfathiazole-resistant mutant which showed no "altered enzyme" but whose resistance they suggested might be due to permeability. Other workers reported no increased uptake of ^{35}S -sulfonamide in chromosomally-resistant organisms (Yegian and Budd, 1945; White and Woods, 1965b). Noll et al (1951) found that sulfathiazole uptake in E. coli was decreased by addition of ρ AB to the uptake medium although there was a limit below which sulfathiazole was taken up even in the presence of high concentrations of ρ AB.

A variety of mechanisms thus appear to function for development of sulfonamide resistance in chromosomal mutants. No one mechanism appears to control resistance.

The development of antibiotic resistance has not been confined to mutations within the chromosome. Resistance may be acquired by the appearance of episomal elements called resistance factors (R factors) in species of the Enterobacteriaceae. The R factor itself consists of (1) a resistance transfer factor (RTF) bearing genes that control the transfer of the factor to a new host, and (2) genetic determinants that

confer drug resistance for one or a number of different antibiotics. Even though episomally drug-resistant bacteria may carry multiple drug determinants, the same mechanism of resistance does not appear to exist for all of the antibiotics concerned.

For example, in chromosomal mutants, chloramphenicol resistance may be due either to altered permeability (Vasquez, 1964) or to structural changes in the 70S ribosome; in episomal mutants, chloramphenicol is inactivated by enzymatic acetylation (Shaw, 1967). Similarly, with aminoglycosidic antibiotics, chromosomal mutants appear to have altered ribosomal binding sites; in episomal mutants on the other hand the antibiotics are enzymatically inactivated by adenylation or phosphorylation reactions.

In the R-factor mediated sulfonamide resistance the degree of resistance is often variable depending upon the properties of the R factor and variations in the susceptibility of host cells (Kabins et al, 1971). This may suggest that multiple mechanisms of resistance exist for episomal as well as chromosomal mutants. Yokota (1964) investigated both chromosomal and episomal mutants of E. coli for differences, if any, that might exist in the mechanism of resistance. He observed a marked increase in intracellular folate levels in chromosomal mutants but relatively little difference in single and multiple R-factor strains as compared to the sensitive parent strain.

Sensitivity of the enzymatic folate system to sulfonamides in episomal mutants but not chromosomal mutants suggested that the "altered enzyme" hypothesis held for the latter, but that permeability might be involved in the R-factor mediated resistance. (Yokota, 1964). Uptake studies with ³⁵S-sulfathiazole supported his hypothesis. Krainski and Unowsky (Bact. Proc. p. 52, 1971) have also demonstrated 9x more sulfonamide uptake by an episomal E. coli mutant than by its parent strain under growing conditions.

The purpose of the present investigation is to study the nature of sulfonamide resistance in a parent strain of E. coli and in a chromosomal and episomal mutant of it. Several of the aforementioned mechanisms have been considered in an attempt to elucidate any differences that exist in the patterns of resistance of these organisms.

MATERIALS AND METHODS

Chemicals

All of the chemicals were obtained commercially and were of reagent grade purity. ρ -Aminobenzoic acid, folic acid and tetrahydrofolic acid were obtained from Calbiochem, Los Angeles, California. The sulfonamides used were a product of Charles E. Frosst Co., Montreal, Quebec.

2- Amino-4-hydroxy-6-hydroxymethyldihydropteridine was synthesized for our use by Raylo Chemical Co., Edmonton, Alberta. Its purity was checked by UV spectrum analysis and found to be approximately eighty percent; its activity was determined by bioautography.

^{35}S -sulfadiazine (S.A.:14.5 mc/mmole) was obtained from the Radiochemical Centre, Amersham, England; ^{14}C - ρAB (S.A.: 10.1 mc/mmole) was obtained from International Nuclear Corporation (INC), Irvine, California.

Pea cotyledon hydrolase was the generous gift of Dr. E. Cossins, Dept. of Botany, University of Alberta, Edmonton.

Bacterial Strains and Cultural Conditions

The strains used for the investigation of sulfonamide resistance were Escherichia coli K-12 and substrains of K-12. The characteristics of these strains are described in Table II.

The stock cultures of all E. coli strains were maintained on ACG slants of the following composition:

TABLE II

Characteristics of E. coli Strains

Strain	Genotype	Minimum Inhibitory Concentration of Sulfonamide (ug/ml)	Origin
K-12	wild type, Su ^S	2	from culture collection of C. H. Pai
K-12-58	bio ⁻ , Su ^S	2	"
K-12-AB-301	met ⁻ , Su ^S	2	"
CSH-2/222	F ⁻ , met ⁻ , pro ⁻ , carries R factor (Su ^R , Sm ^R , Cm ^R , Tc ^R)	>1000	from T. Watanabe, Keio University, Tokyo, Japan
AB 1932/E521	F ⁻ , arg ⁻ , met ⁻ , carries R factor (Su ^R , Sm ^R , Cm ^R , Tc ^R)	>1000	from D. H. Smith, Chil- dren's Hospital Medical Centre, Boston, Mass.
K-1258/222	bio ⁻ , carries R factor (Su ^R , Sm ^R , Cm ^R , Tc ^R)	>1000	R factor 222 transferred by conjugation from CSH-222 to K-12-58.

TABLE II (Continued)

Strain	Genotype	Minimum Inhibitory Concentration of Sulfonamide (ug/ml)	Origin
K-12-AB- 301/222	met ⁻ , carries R factor (Su ^R , Sm ^R , Cm ^R , Tc ^R)	>1000	R factor 222 transferred by conjugation from CSH-2/222 to K-12-AB-301.
K-12-AB- 301/E521	met ⁻ , carries R factor (Su ^R , Sm ^R , Cm ^R , Tc ^R)	>1000	R factor E521 transferred by conjugation from 1932/ E521 to K-12-AB-301.
K-12-58-3	bio ⁻ , Su ^R	>1000	NTG mutagenesis of K-12-58; isolated as Sd ^R mutant.
K-12-AB- 301-5	met ⁻ , Su ^R	>1000	NTG mutagenesis of K-12-AB-301; isolated as Sd ^R mutant.
K-12-AB- 301-8	met ⁻ , Su ^R	>1000	NTG mutagenesis of K-12-AB-301; isolated as Sth ^R mutant.

Yeast extract (Difco)	10 g.
Casitone (Difco)	10 g.
K_2HPO_4	5 g.
Glucose	5 g.
Agar	16 g.
Distilled water	to 1000 ml.

For maintenance of strains K-12-58-3, K-12-AB-301-5 and K-12-AB-301-8, ACG slants were supplemented with sulfadiazine (1000 ug/ml); for strains carrying R factors, the slants were supplemented with sulfadiazine (100 ug/ml), tetracycline (25 ug/ml); chloramphenicol (30 ug/ml) and streptomycin (10 ug/ml).

Inoculum cultures were prepared in ACG broth.

The medium used for the growth of E. coli was a modification of that of Davis & Mingioli (1950) and contained the following ingredients:

K_2HPO_4	7.0 g.
KH_2PO_4	3.0 g.
$(NH_4)_2SO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	0.1 g.
Glucose	2.0 g. (added aseptically)
Distilled water	to 1000 ml.
pH	7.0

For studies on the inhibition of growth by sulfonamides, Davis - Mingioli medium was supplemented with purified Difco Bacto Vitamin-free Casamino Acids (2.8 g./l.). The casamino acids were purified by adding 3 g of Norit A charcoal to the vitamin solution (14 g./100 ml; pH 3.5), stirring for 1 HR. at room temperature, filtering and storing in the frozen state. For studies on the synthesis of folate derivatives by auxotrophic mutants, DL-methionine (50 ug/ml.) or d-biotin (10 ug/ml.) was added aseptically.

All flask cultures were incubated at 37°C on a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) maintained at a speed of 200 rpm; inoculum cultures were incubated at 37°C on a tube roller (Tissue Culture Rollordrum, model TC5, New Brunswick Scientific Co., New Brunswick, N.J.).

Isolation of Sulfonamide-Resistant Mutants of *E. coli*

(a) Chemical mutagenesis: Mutagenesis with N-methyl-N¹-nitro-N-nitrosoguanidine (NTG) was carried out according to the method of Adelberg et al. (1965). The double-strength basal media used throughout the mutagenesis contained, per liter of distilled water:

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	16.4 g.
KH_2PO_4	5.4 g.
$(\text{NH}_4)_2\text{SO}_4$	2.0 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g.

Ca(NO₃)₂ 10 mg.

FeSO₄ · 7H₂O 0.50 mg.

pH 7.2

Glucose 4 g. (added
aseptically)

Inoculum cultures were prepared in nutrient broth containing, per liter of distilled water:

tryptone 10 g.

Yeast extract (Difco) 5 g.

NaCl 10 g.

pH 7.2

Refrigerated log-phase cultures of E. coli K-12-58 and K-12-AB-301 were diluted with fresh nutrient broth to about 1×10^6 cells/ml. and incubated at 37°C with shaking until cultures were in logarithmic phase; cells were filtered on a 47 mm. Millipore filter, pore size 0.45 μ and washed 3 times with 5-10 ml sterile Tris-maleic acid (TM) buffer 0.05 M, pH 6.0. The cells were suspended by placing filter membrane in 10 ml. of sterile TM buffer in a 125 ml. Erlenmeyer flask and agitating on a Vortex Junior Mixer. After membrane was removed, NTG was added at a final concentration of 100 ug/ml. and the treatment mixture incubated in a gyrotory water bath shaker (N.B. Scientific Co., New Brunswick, N.J.) at 180 rpm. at 37°C for 30 minutes. A 1.0 ml. sample was removed, filtered, washed 3 times with 5 ml. aliquots of cold basal media. After 12 hr. of incubation at 37°C, a 10% (v/v) inoculum was transferred to fresh basal media containing

sulfonamide (100 ug/ml) incubated for 24 hr. at 37°C and diluted onto basal agar plates containing glucose and concentrations of sulfonamide ranging from 0 - 1000 ug/ml. Colonies growing on the highest concentration of sulfonamide were picked up and transferred to fresh basal media containing sulfonamide (in concentrations similar to that on plates). After overnight growth, the cultures were re-streaked on minimal agar plates (described on page 28) to confirm the original nutritional requirements of the strains.

(b) Transfer of R factors by conjugation. The transfer of R factors by conjugation was performed according to the procedure of Watanabe and Fukasawa (1960). After conjugation was allowed to occur, cells were plated on minimal media (Section 2) containing chloramphenicol (30 ug/ml.); tetracycline (30 ug/ml.); streptomycin (10 ug/ml.) and sulfadiazine (1000 ug/ml.). After incubation at 37°C for 40 hr., colonies appearing on test plates were picked with a needle, and transferred to fresh minimal media supplemented with sulfadiazine. The isolates were examined for the original nutritional requirements and resistance levels.

Measurement of Sulfonamide Sensitivity

Varying amounts of sulfonamide were added aseptically to sterile test tubes (18 x 150 mm.) and the volumes adjusted to 2.5 ml. with sterile distilled water. Double-strength Davis-Mingioli minimal media seeded with a test strain of E. coli was dispensed in 2.5 ml. aliquots into the above tubes.

The tubes were incubated on a tube roller which was rotated at a speed of 60 rpm. at 37°C for 24 hr. Growth was measured in a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., Inc., New York) fitted with a No. 60 filter.

E. coli inoculum for the study was grown in ACG broth at 37°C for 8-10 hr., washed 3 times with sterile distilled water and resuspended to an absorbance of 0.4 at a wavelength of 600 nm. using a Spectronic 20 (Bausch and Lomb, Rochester, N.Y.) spectrophotometer with a light path of 1.3 cm. One ml. of the inoculum was used per 100 ml. of double strength media.

Preparation of Folate-Synthesizing Enzymes

All steps in the preparation of enzyme fractions were carried out at 0-4° C.

(a) Cell-free extracts: Cells were grown in Davis - Mingioli media at 37°C for 12 hr., harvested by centrifugation at 8000 g for 10 min. and washed 2 times with tris (hydroxymethyl) aminomethane (Tris) - hydrochloride (50 mM, pH 8.0). The cells were resuspended in the same buffer to a concentration of 50 mg. dry weight/ml., placed in a stainless steel cup and immersed in an ice bath. Cell-free extracts were obtained by sonic disintegration using a Biosonik III (Bronwill Scientific Co., Rochester, N.Y.), followed by centrifugation at 27,000 g. for 45 min.

(b) Protamine sulfate treatment: Three (3) milliliters of protamine sulfate solution (2%, pH 5.0) were added per 8 ml.

of cell-free extract. The mixture was made 0.01 M with respect to 2-mercaptoethanol and stirred for 30 mins. The precipitate was removed by centrifugation at 12,000 g for 20 min.

(c) Ammonium sulfate fractionation: To the supernatant solution obtained from the protamine sulfate treatment was added ammonium sulfate (20% saturation) with stirring for 30 min. After the precipitate was removed by centrifugation at 12,000 g. for 20 min., an additional amount of ammonium sulfate was added (60% saturation) and stirring continued for 30 min. Upon centrifugation for 20 min. at 12000 g., the precipitate was recovered and dissolved in a minimal amount of Tris-hydrochloride (50 mM, pH8.0) containing 2-mercaptoethanol (10 mM).

(d) Dialysis: The protein solution obtained by 20-60% ammonium sulfate fractionation was dialyzed against 1000 volumes of Tris-hydrochloride (50 mM, pH 8.0) containing 2-mercaptoethanol (10mM) at 4°C for 16-20 hours.

Assay of the Folate-Synthesizing Enzyme System

The activity of the folate-synthesizing enzyme system was measured by determining the amount of folate equivalents formed in the presence of pAB and DHP.

Unless otherwise specified, the reaction mixture contained the following components in a final volume of 200 μ l.:

p - aminobenzoic acid	0.05 mM
dihydropteridine [*]	0.04 mM
MgCl ₂	5.0 mM
ATP	0.1 mM
Tris-hydrochloride (pH 8.0)	100 mM

* prepared in 67 mM 2-mercaptoethanol.

The reaction mixture was prepared in polyethylene micro-test tubes and pre-incubated at 30°C for 5 min. in a water bath (Temp. Bloc Module Heater, Lab-line Instruments Inc., Illinois). The reaction was started by addition of the dialyzed enzyme preparation (~ 0.4 mg/200 ul.); the tubes were incubated at 30°C for 90 mins. after which time the reaction was stopped by placing the tubes in an acetone-dry ice bath for 5 sec. In enzyme assays where folate rather than pterate was to be measured, L-glutamic acid (0.05 mM) was added to the reaction mixture.

For experiments designed to determine the optimum conditions for the enzyme assay, the incubation temperature used was 37°C (Fig. 8 to 14).

Enzyme activity was determined by microbiological assay of the amount of folic acid equivalents formed, using S. faecalis as assay organism. Specific activity was expressed as nanomoles of folate equivalents synthesized per milligram of protein per minute under the assay conditions described.

Microbiological Assays

(a) Folic acid and its derivatives. Folic acid and its derivatives were measured by microbiological assay employing two different organisms, Streptococcus faecalis ATCC 8043 and

Lactobacillus casei ATCC 7469.

(1) S. faecalis assay -

Maintenance of stock cultures: S. faecalis was maintained in Micro Inoculum Broth (Difco), transferred daily and incubated at 30°C.

Preparation of inoculum: A 24-hour culture of S. faecalis grown at 30°C in 5 ml. Micro Inoculum Broth (Difco) was washed 3 times and resuspended in 15 ml. of sterile distilled water. One ml. of inoculum suspension was used to seed 200 ml. of double-strength Folic Acid Assay Medium (Difco).

Assay procedure: To duplicate test tubes (18 x 150 mm.) were added 1.8 ml. or less of assay material and the total volume adjusted to 2.5 ml. with ascorbic acid solution (12 mg/ml., pH 7.0). The tubes were autoclaved for 7 min. at 121°C and 15 psi and cooled immediately in an ice-water bath. To each tube was added 2.5 ml. of sterile double-strength assay medium seeded with S. faecalis; the tubes were incubated at 30°C for 18 hr. Growth was measured turbidimetrically in a Klett-Summerson Colorimeter fitted with a No. 66 filter. The colorimetric readings were converted to nannograms of folate equivalents by means of a standard curve prepared for each assay.

A folic acid stock solution (10 µg/ml. in 0.1 N NaOH) was diluted to 2 ng/ml. for each assay and used in preparation of a standard curve (Fig. 2). The procedure for setting up a standard curve was identical to that used for preparation of unknown samples.

(2) L. casei assay -

Maintenance of stock cultures: L. casei was maintained on Yeast extract acetate-glucose agar slants, transferred weekly, incubated at 37°C for 24 hr. and refrigerated. The composition of the stock media was as follows:

Yeast extract	5 g.
Glucose	1 g.
Sodium acetate • 3H ₂ O	1 g.
Agar	3 g.
Distilled water	to 200 ml.

The media was dispensed into tubes in 7 ml. aliquots and autoclaved for 20 min. at 121°C.

Preparation of inoculum: The medium used for the preparation of inoculum was of the following composition:

Peptone	2 g.
Yeast extract	0.4 g.

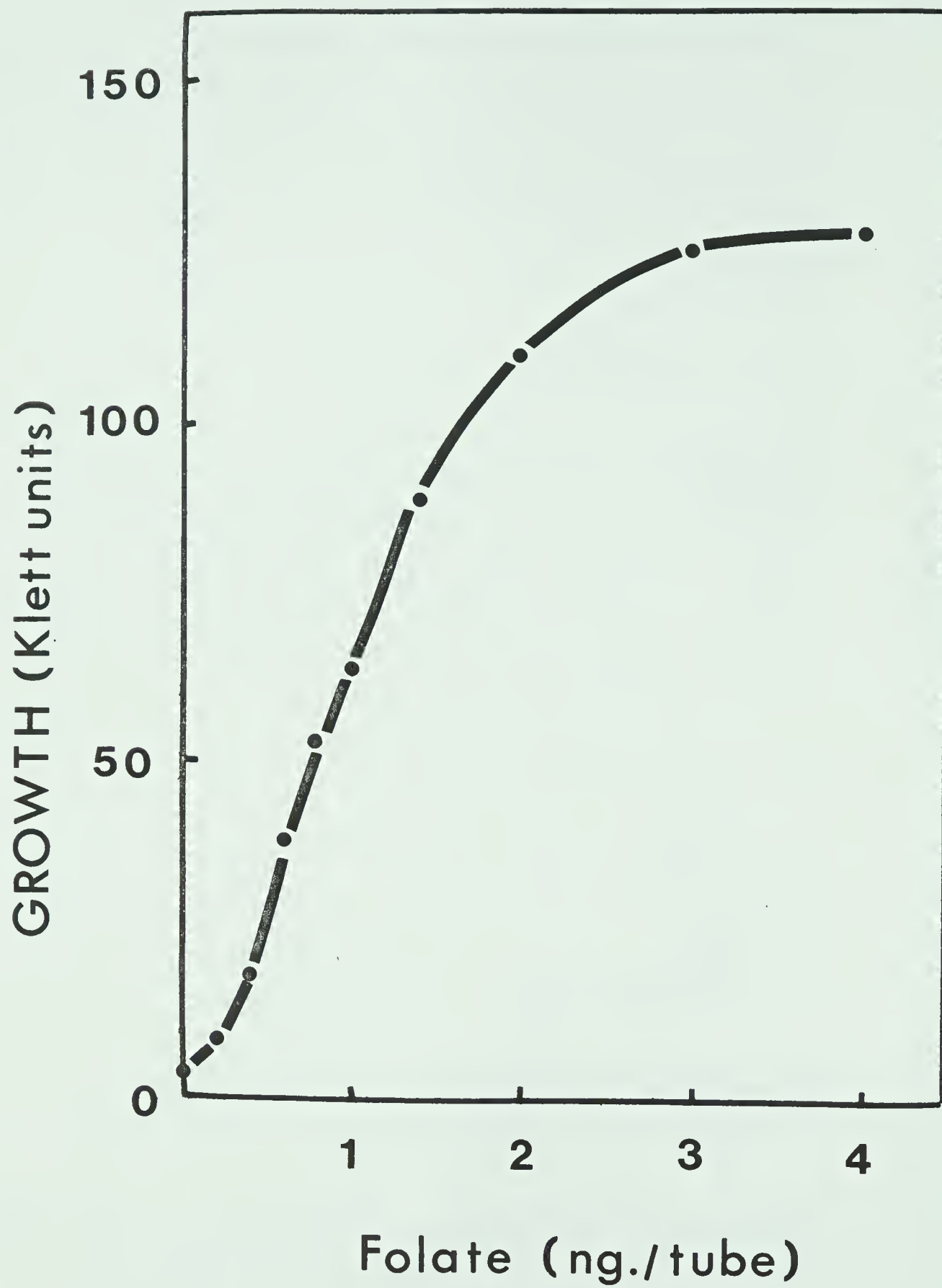


FIGURE 2

Standard curve relating concentration of folic acid to growth of S. faecalis 8043. Turbidity was measured in a Klett-Summerson photoelectric colorimeter after 18 hours incubation at 30°C.

sodium acetate	4 g.
glucose	4 g.
salt solution A [*]	2 ml.
salt solution B ^{**}	2 ml.
Distilled water	to 500 ml.
pH	6.8

* Salt solution A - 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 were dissolved in distilled water and subsequently diluted to 500 ml.

** Salt solution B - 10 g. of $MgSO_4 \cdot 7H_2O$ and 0.5 g. each of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were dissolved in distilled water, 0.5 ml. concentrated HCl was added and the solution diluted to 500 ml. with distilled water.

Both solutions were stored under toluene

The media was dispensed into tubes in 5 ml. aliquots and autoclaved for 10 min. at 121°C and 15 psi.

An 18 hr. culture of L. casei grown in the inoculum medium at 37°C was washed 3 times and resuspended in 30 ml. of sterile distilled water. 0.1 ml. of inoculum suspension was used to seed 100 ml. of double-strength Folic Acid Casei Assay Media (Difco).

Assay Procedure: The procedure used for the L. casei assay system was similar to that used for the S. faecalis assay with the following modifications. Test tubes with 16 x 150 mm. dimensions were used and the total volume of assay mixture was 10 ml. Folic Acid Casei Medium (Difco) was used as assay medium, and the incubation was carried out at 37°C for 20 hr.

A typical standard curve is shown in Fig. 3.

(b) p-Aminobenzoic acid: Lactobacillus plantarum ATCC 8014, previously described as L. arabinosus, was employed for the microbiological assay of p-aminobenzoic acid.

Maintenance of stock cultures: L. plantarum was maintained on APT (Difco) agar slants, transferred monthly and incubated at 30°C.

Preparation of inoculum: Five (5.0) ml. of an 18 hour culture of L. plantarum, grown at 30°C in modified Wright-Skeggs medium (Wright & Skeggs, 1944) containing 5×10^{-4} µg. biotin per ml., was washed 3 times and resuspended in 15 ml. of sterile distilled water. 0.1 ml. of inoculum suspension was used to seed 100 ml. of sterile double-strength assay medium.

Assay procedure: The media described by Wright and Skeggs (1944) was modified and employed as

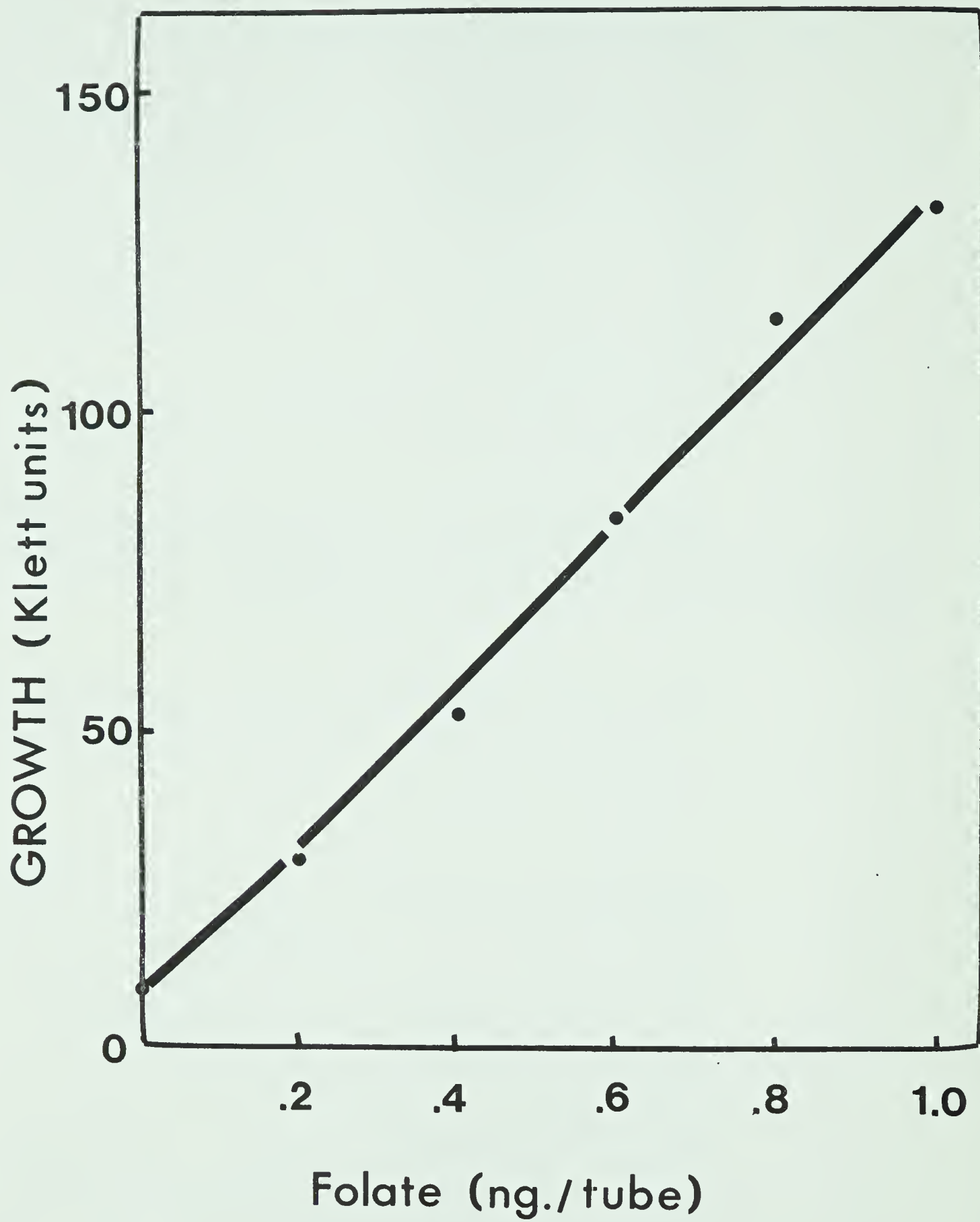


FIGURE 3

Standard curve relating concentration of folic acid to growth of L. casei. Turbidity was measured in a Klett-Summerson photoelectric colorimeter after 20 hours incubation at 37°C.

assay media. The composition of double-strength assay media is as follows:

Vitamin-free casamino acids	18 g.
Sodium acetate $\cdot 3\text{H}_2\text{O}$	22 g.
DL-tryptophan	600 mg.
L-cysteine-HCL $\cdot \text{H}_2\text{O}$	450 mg.
K_2HPO_4	1.5 g.
KH_2PO_4	1.5 g.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	600 mg.
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	30 mg.
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	30 mg.
Adenine sulfate	10 mg.
Guanine-HCl	10 mg.
Xanthine	10 mg.
Uracil	10 mg.
Thiamine-HCl	3 mg.
Ca pantothenate	3 mg.
Pyridoxine-HCl	6 mg.
Riboflavin	3 mg.
Nicotinic acid	3 mg.
Biotin	0.5 ug.
Glucose (added aseptically)	40 g.
H_2O	to 1000 ml.
pH	6.8

One ml. or less of assay material was added in duplicate into test tubes (16 x 150 mm) and the

total volume adjusted to 5 ml. with distilled water. The tubes were autoclaved for 10 min. at 121°C and 15 psi and cooled immediately in an ice-water bath. To each tube was added 5 ml. of sterile double-strength assay medium seeded with L. plantarum. The tubes were incubated at 30°C for 20 hr. Growth was measured turbidimetrically in a Klett-Summerson Colorimeter fitted with a No. 66 filter. The colorimeter readings were converted to ng. of p-aminobenzoic acid by means of a standard curve (Fig. 4).

Analysis of Folates in E. coli K-12 Strains

Folic acid content of bacterial cultures was measured by microbiological assays using either S. faecalis or L. casei or both.

Folic acid and its derivatives were assayed in two different forms - extracellular and intracellular folate.

(a) Extracellular folate. Folic acid and its derivatives present in the supernatant fluid of a culture, plus those removable from cells by washing were defined as extracellular folate.

Supernatant fluid was obtained by centrifugation of a growing culture at 8000 g for 10 min. Cells were washed 2 times with Tris-HCl (50 mM, pH 8.0) containing ascorbate (12 mg/ml.) and the washing medium added to the supernatant

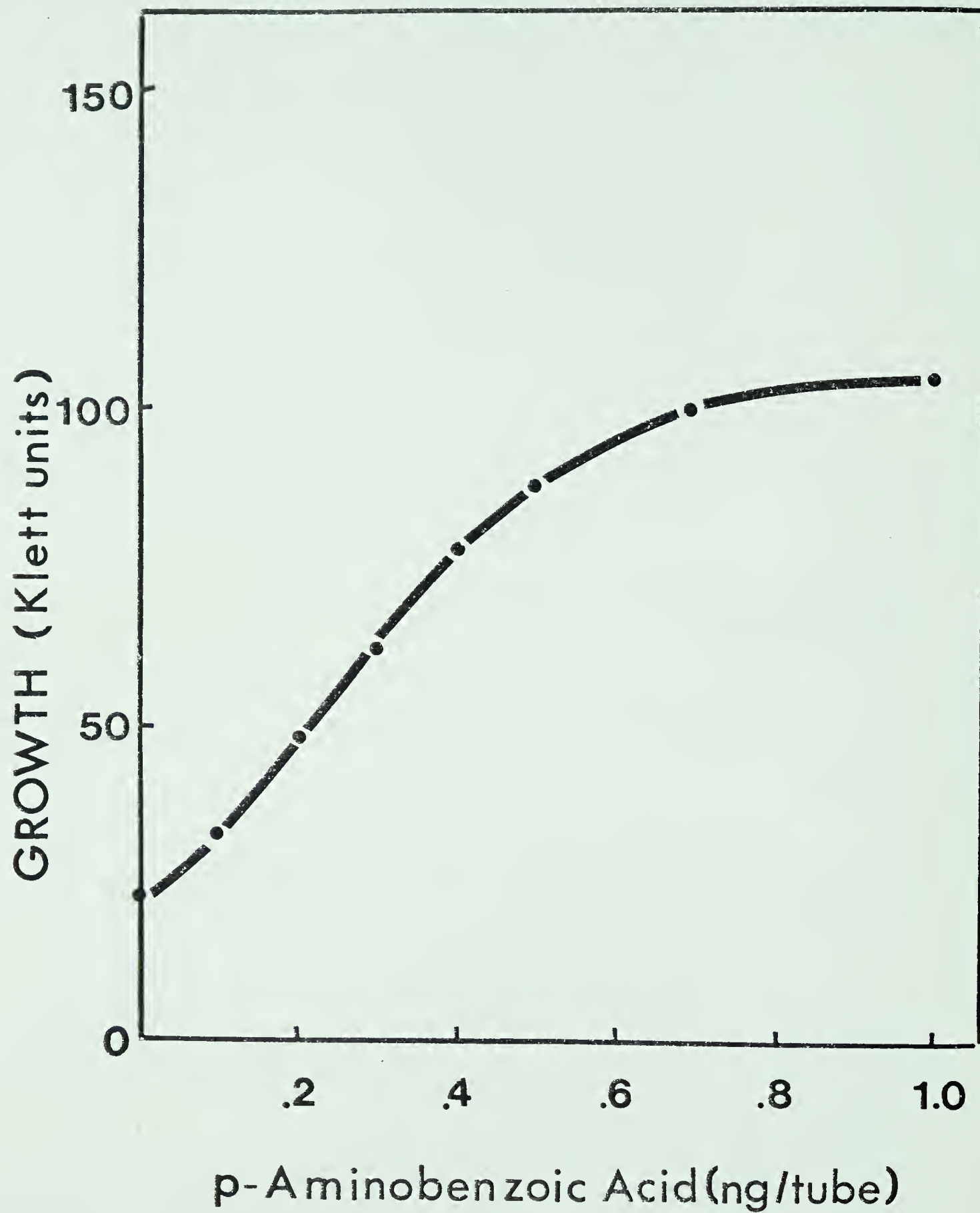


FIGURE 4

Standard curve relating concentration of ρ -aminobenzoic acid to growth of L. plantarum. Growth was measured in a Klett-Summerson photoelectric colorimeter after 20 hours incubation at 30°C.

fluid for microbiological assay of extracellular folate.

(b) Intracellular folate. Folate and its derivatives assayable after acid or enzymatic hydrolysis of cells were defined as intracellular folate.

After cells were washed 2 times with Tris-hydrochloride (50 mM, pH8.0) containing ascorbate (12 mg/ml.), they were resuspended in fresh buffer plus ascorbate to a concentration of 50 mg. dry weight per ml.

(1) Acid hydrolysis - to 0.1 ml. of the cell suspension was added 0.5 ml. of 6N HCL; the cells were hydrolysed at 121°C and 15 psi for 15 mins., cooled quickly in an ice bath and neutralized to pH 7 ± 1.0 with 6N NaOH. The acid hydrolysate was diluted to 10 ml. with distilled water and centrifuged at 27,000 g for 30 min. to remove cell debris.

(2) Enzymatic hydrolysis - cells were broken by sonic disintegration and centrifuged at 27,000 g for 45 min. to remove cell debris. The supernatant fluid was placed in a boiling water bath for 5 min., cooled quickly and centrifuged at 12,000 g for 20 min. The pH of the supernatant fluid was adjusted to 4.5 with 0.5 N HCl. Samples containing 20-50 mg. folate equivalents were placed in test tubes (18 x 150 mm). One ml. of a 1:10 dilution of pea cotyledon hydrolase preparation

was added and the volume adjusted to 10 ml. with acetate buffer (0.1 M, pH 4.5). Contents of the tube were mixed and incubated in a water bath at 35°C for 2-3 hrs. The hydrolysates were then stored at 4°C until contents were assayed.

Analysis of ρ -Aminobenzoic Acid in Bacterial Cultures

The ρ -aminobenzoic acid content of bacterial cells was measured by microbiological assay using L. plantarum as assay organism.

Cells were grown overnight at 37°C in Davis-Mingioli medium supplemented with methionine, centrifuged at 8000 x g for 10 min. and washed 2 times with Tris-HCl (0.5 M, pH 8.0). Cells were suspended to a concentration of 200 mg. dry weight per ml. Two ml. of suspension were boiled for 10 min, cooled quickly in an ice-bath and centrifuged at 27,000 x g for 10 min. The supernatant fluid was retained for ρ AB analysis. The cell debris was washed 2 times with Tris-HCl (0.05 M, pH 8.0) and the washes analysed for ρ AB content.

Uptake of ρ -Aminobenzoic Acid - ^{14}C

Preparation of cells: Cultures of E. coli grown overnight at 37°C in Davis-Mingioli media were centrifuged at 8000 x g for 10 min., washed once with Tris-hydrochloride (100 mM, pH 8.0), resuspended to a concentration of 0.67 mg. dry weight per ml. and placed in an ice bath until used. Cell suspension was pre-incubated at 37°C for 5 min. prior to uptake.

Preparation of uptake menstrum: One ml. of Davis-Mingoli media containing 0.2 uc of ^{14}C - ρAB was added to an acid-washed 50 ml. Erlenmeyer flask. The mixture was pre-incubated at 37°C in a gyrotory water bath shaker at a speed of 180 rpm for 5 min.

Uptake procedure: One ml. of the cell suspension was added to the uptake menstrum described above. One hundred microliter samples of uptake mixture were removed in duplicate at various time intervals for radioactivity counting.

When the effect of sulfonamide on the uptake of ρAB was examined, sulfadiazine was added to the uptake menstrum prior to the addition of cells.

Preparation of samples for counting: Millipore filter discs (diameter: 25 mm; pore size: 0.45 μ) were wetted in distilled water and placed in Millipore filter holders. The filters were pre-washed with 10 ml. of cold Tris-hydrochloride (0.1 M, pH 8.0) and 5 ml. of the same buffer added to the reservoir head. Samples removed at various time intervals were diluted into the buffer head, filtered and washed two times with 10 ml. aliquots of cold buffer. The filters were dried under an infra-red lamp and placed in scintillation vials containing 5 ml. of toluene scintillation fluid. The composition of the scintillation fluid was as follows:

PPO	4 gm.
dimethyl POPOP	50 mg.

[1,4-bis-2-(4-methyl-5-phenyloxazolyl)- benzene]

Toluene

to 1 liter.

Radioactivity was measured in a liquid scintillation counter (Mark I, Nuclear Chicago) using channel ratio methods to determine the counting efficiency of the instrument.

Uptake of ^{35}S -sulfadiazine

Preparation of cells, uptake menstium and sample preparation were essentially the same as for the ρ -aminobenzoic acid - ^{14}C uptake experiment. Sulfadiazine was added to the uptake menstium at concentrations of 100, 200 and 500 $\mu\text{g./ml.}$ (S.A. = 0.058 $\mu\text{c}/\mu\text{g.}$). Uptake was also measured in the presence of 10^{-4} M ρAB , and 2.2×10^{-2} mM folate. Radioactivity was measured in toluene using a liquid scintillation counter, and using a planchet counter (Nuclear Chicago).

Paper Chromatography

Identification of the products of the folate-synthesizing enzyme system was made using descending paper chromatographic techniques.

Whatman No. 1 paper was saturated with a solution of ascorbate (12 mg/ml, pH 7.0) and dried. Samples were applied in the presence of hydrogen gas to prevent oxidation of reduced compounds. Chromatograms were developed in phosphate buffer (0.01 M, pH 7.0) containing ascorbate (6 mg/ml.) This was a modification of the method used by Brown et al. (1961).

Bioautography

Folic acid assay medium (Difco) containing 1.5% agar was autoclaved at 121°C and 15 psi for 10 min. The media was cooled to 45°C and 2,3,5 - triphenyltetrazolium chloride (0.05 g./liter) was added as growth indicator. One ml. of S. faecalis inoculum prepared as outlined in Part 7, Material and Methods, was used to seed 600 ml. of assay medium. After the bioautographic plate was poured and solidified, the paper chromatogram containing the separated folate derivatives was placed on the agar plate for 30 min. to allow compounds to diffuse into the assay media. The bioautogram was incubated at 30°C for 18 hr. Spots of folate derivatives were revealed by areas of growth augmented by the pink-red colour of the tetrazolium dye.

Sulfonamides were identified by bioautography after chromatographic separation in butanol: acetic acid: water (50:15:60) solvent. E. coli K-12-AB-301 was used as inoculum and Davis-Mingioli medium as the growth medium. Spots of sulfonamides were identified by areas of growth inhibition revealed as clear areas against a pink-red background.

EXPERIMENTAL RESULTS

Effect of Sulfonamides on Growth

The growth of sulfonamide-sensitive (K-12-AB-301) and resistant (K-12-AB-301-5 and K-12-AB-301/222) strains of E. coli was followed in the presence or absence of sulfadiazine (Fig. 5). The growth rate of the sensitive strain was altered by the presence of sulfadiazine at a concentration of 10 ug/ml. (Fig.5A), whereas the growth of resistant strains was affected only slightly at a concentration of 500 ug/ml. The chromosomal mutant (K-12-AB-301-5) had a generation time of 51 min. in the absence of sulfadiazine and 56 and 68 min. in the presence of sulfadiazine concentrations of 250 and 500 ug/ml. respectively (Fig. 5B). A similar degree of resistance was shown with the episomal mutant, K-12-AB-301/222 (Fig. 5C). Moreover, it was observed that cultures of the resistant strains grown in the presence of sulfadiazine reached the same turbidity in the stationary phase as did cultures grown in sulfadiazine-free media.

The above experiments were carried out with shaking flask cultures using a relatively high inoculum concentration (absorbance at zero time 0.03). When sulfonamide sensitivity was examined with rotating tube cultures using a low inoculum concentration (absorbance at zero time: 0.002), the difference in the degree of sulfonamide sensitivity in sensitive and resistant strains became more pronounced. With a concentration

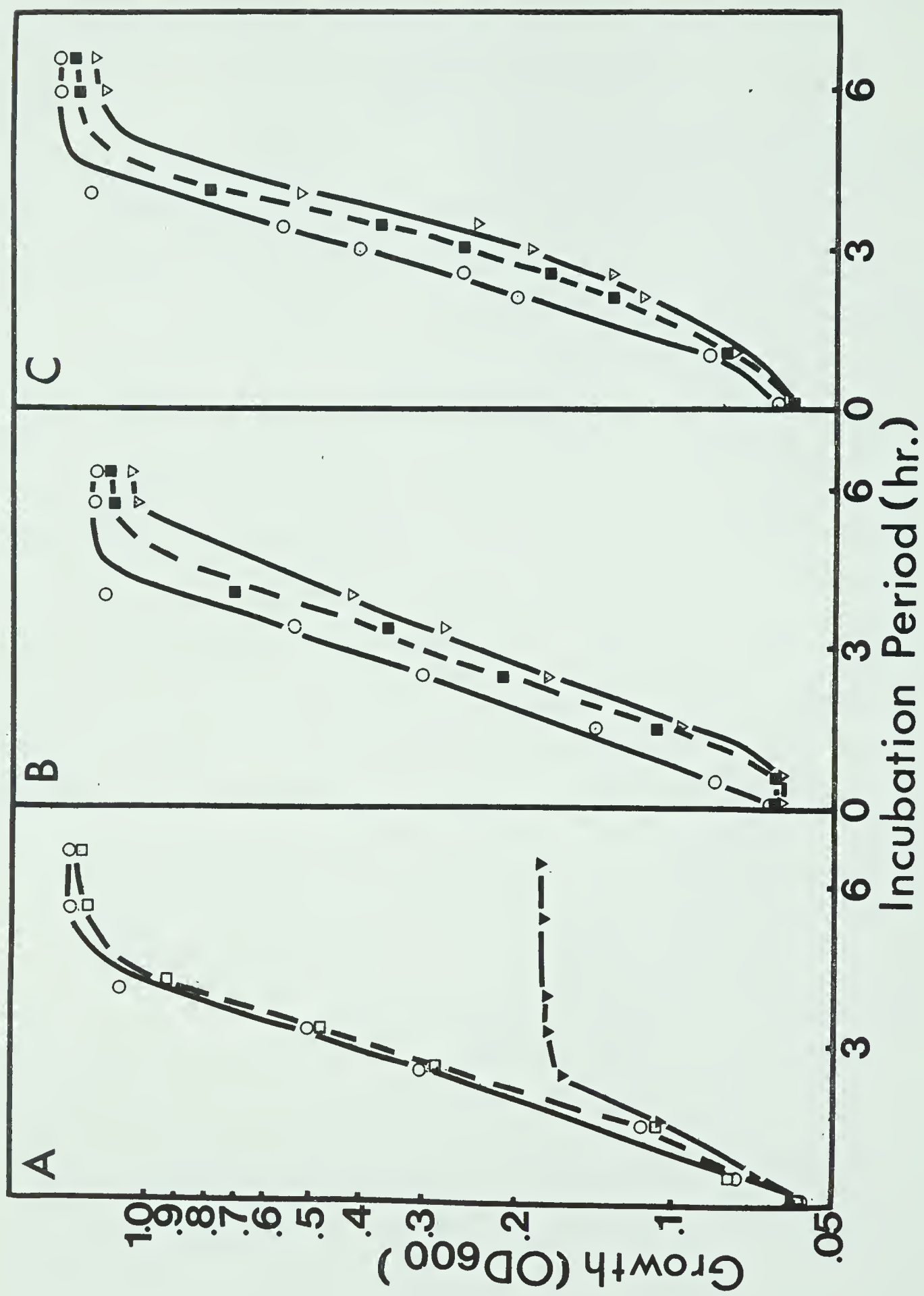


FIGURE 5

EFFECT OF SULFADIAZINE ON THE GROWTH PATTERNS
OF E. COLI K-12 SUBSTRAINS

- A. E. coli K-12-AB-301
- B. E. coli K - 12-AB-301-5
- C. E. coli K - 12-AB-301/222

Inoculum cultures of all strains were prepared in Davis-Mingioli medium with added methionine. Inoculum cultures were grown overnight at 37°C, and sufficient inoculum added to one liter of fresh Davis-Mingioli medium to give an initial absorbance of 0.06. Various concentrations of sulfadiazine were added; the flasks were incubated at 37°C on a gyrotory shaker set at 200 rpm.

LEGEND:

- No sulfadiazine (Sd) added
- 250 ug/ml. Sd added
- ▽—▽ 500 ug/ml. Sd added
- 1.0 ug/ml. Sd added
- ▼—▼ 10 ug/ml. Sd added

of 1.0 ug/ml. sulfadiazine, the growth of a sensitive strain (K-12-58) was only 66% of that obtained in the absence of sulfadiazine; however, the growth of resistant strains (K-12-58-3 and K-12-58/222) was affected only very slightly with sulfonamide concentrations as high as 1500 ug/ml. (Fig.6).

The observation that the growth of sulfonamide resistant strains was not affected to any great extent by high concentrations of sulfadiazine led to an investigation of the inhibition of growth of different E. coli K-12 substrains by various sulfonamides (Table III). The "tube method" as described in the Materials and Methods section was used for the study; the degree of sensitivity was expressed in terms of a "50% growth inhibition level," defined as that concentration of sulfonamide at which the growth of an organism is limited to one-half of the growth which occurs in the absence of sulfonamide.

It was observed that the 50% inhibition levels varied with the sulfonamide under test. Using sulfadiazine or sulfamerazine, the episomal and chromosomal mutants were about 500 times more resistant than were the sensitive strains; using sulfathiazole and sulfamethazine. the resistance was 100 to 400 times greater in the resistant strains than in the sensitive strains. Two strains of chromosomal mutants (K-12-AB-301-5 and K-12-AB-301-8), which had been isolated against sulfadiazine and sulfathiazole respectively, were equally resistant to all sulfonamides tested.

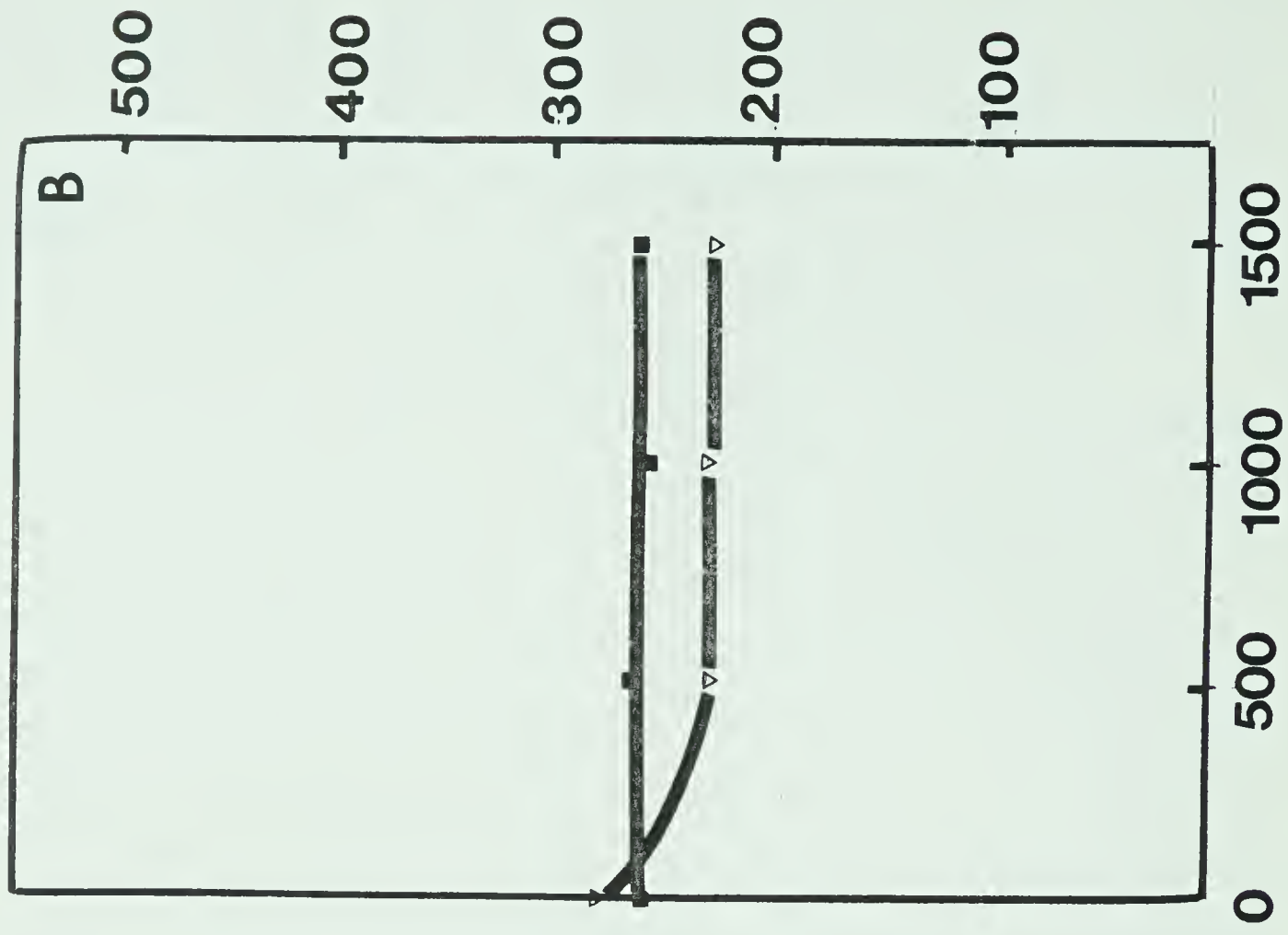
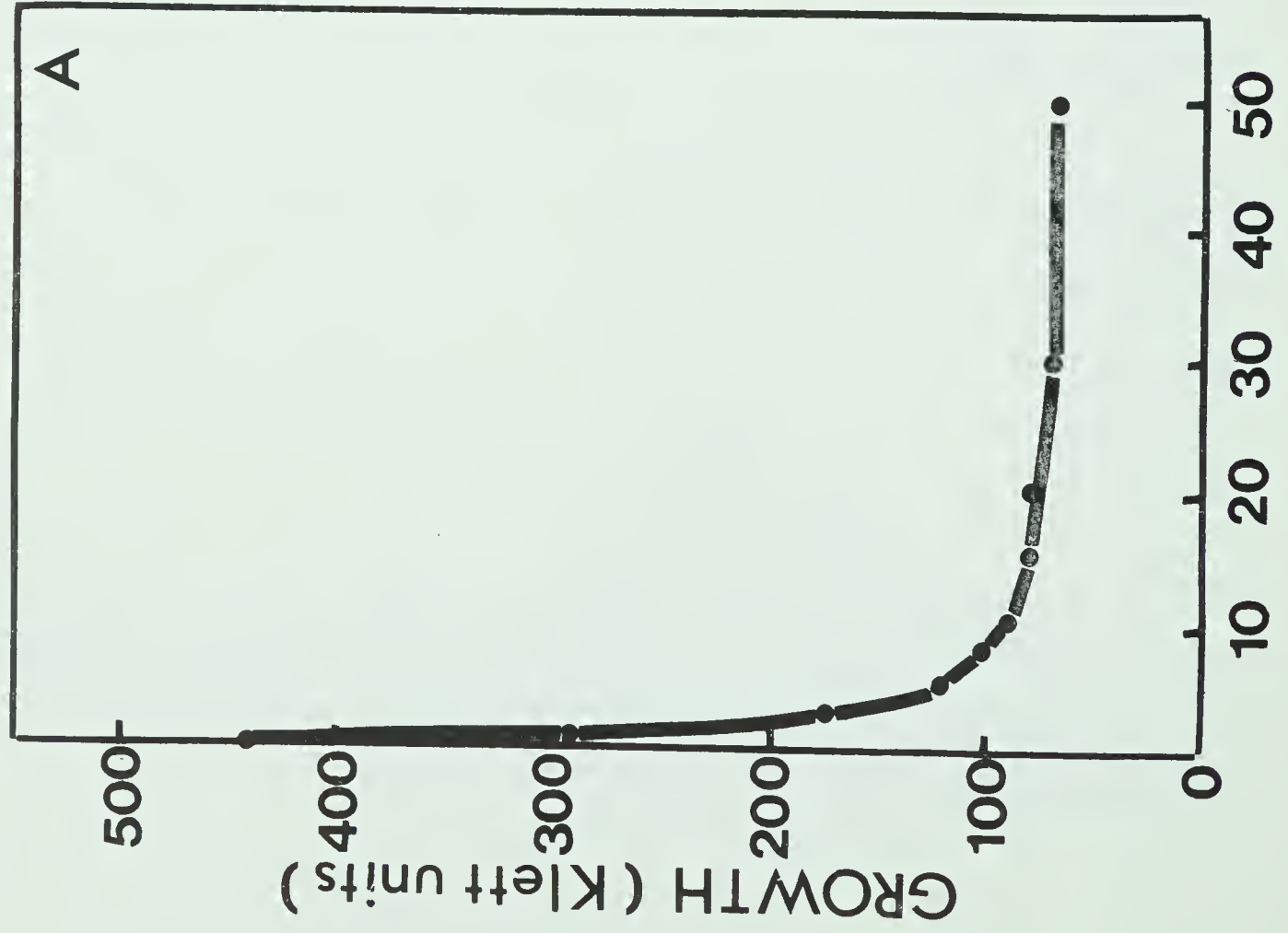


FIGURE 6

EFFECT OF SULFADIAZINE CONCENTRATION ON THE GROWTH
OF E. COLI K-12 SUBSTRAINS

Varying sulfadiazine concentrations were added aseptically to sterile 18 mm. test tubes and diluted to 2.5 ml. with distilled water; 2.5 ml. double-strength Davis-Mingioli medium inoculated with a washed suspension of bacterial culture (1 ml. of absorbance = 0.40 per 100 ml. media) was added. Tubes were incubated 24 hr. on a tube roller at 37°C. Growth was measured with a Klett-Summerson colorimeter fitted with #66 filter.

LEGEND:

- - E. coli K-12-58
- ▽—▽ - E. coli K-12-58/222
- - E. coli K-12-58-3

TABLE III

Inhibition of Growth of E. coli K-12 Substrains
by Various Sulfonamides

Strain	50% Growth Inhibition Level (mM) .			
	Sulfadiazine	Sulfathiazole	Sulfamerazine	Sulfamethazine
Sensitive Parent				
K-12-AB-301	0.004	0.010	0.010	0.028
K-12-58	0.008	0.015	0.013	0.052
Episomal mutants				
1932/E521	>4.0	1.4	4.9	2.8
K-12-58/E521	>4.0	3.8	4.0	3.3
Chromosomal mutants				
CSH-2/222	>4.0	1.8	2.3	1.9
K-12-AB-301/222	>4.0	3.6	>4.0	3.8
K-12-58/222	>4.0	4.0	>4.0	4.4
Chromosomal mutants				
K-12-AB-301-5	>4.0	3.7	>4.0	4.0
K-12-AB-301-8	>4.0	>4.0	>4.0	>4.0
K-12-58-3	>4.0	4.0	>4.0	4.5

Synthesis of Folate Compounds in Cell-Free Extracts

(a) Requirements of the reaction. The dialyzed preparations of ammonium sulfate fractions (20-60% cut) were used as the source of the folate-synthesizing enzyme in the investigation of the requirements for enzymatic synthesis of folic acid. As illustrated in Table IV, the essential components of the reaction were:

- (1) 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine.
- (2) p-aminobenzoic acid
- (3) glutamate
- (4) ATP

The oxidized form of the pteridine, 2-amino-4-hydroxy-6-hydroxymethylpteridine, was inactive as substrate and p-aminobenzoyl-glutamate could readily replace p-aminobenzoic acid and glutamate. In the presence of pABG above, almost as much S. faecalis-assayable folate compound was formed as in the presence of pAB and glutamate. As described in a later experiment (Fig. 7) this was found to be due to formation of pteronic acid, a compound that is as active as folic acid in supporting the growth of S. faecalis.

(b) Identification of reaction products. The products of the enzyme reaction were examined by bioautographic technique (Fig. 7). Distinct separation of folate and pterate was possible. The R_f values of folate and pterate were 0.33 and

TABLE IV

Requirements of the Folate-Synthesizing Enzyme System
of E. coli K-12 Substrains

Conditions	Specific Activity (nmoles folate) equivalents formed/mg protein/min)x 10 ⁴	
	Experiment 1	Experiment 2
a Complete	8.2	57
minus enzyme	<0.3	
minus DHP	<0.3	
minus DHP; plus pteridine	<0.3	
minus ρAB		<4.0
minus glutamate	7.1	
minus ρAB, glutamate	0.6	
minus ATP	1.3	
minus ρAB, glutamate; plus ρABG	6.7	
b Zero time control	1.3	

a Complete system - as described in Materials and Methods except that glutamate (0.05 mM) was added.

b Zero time control - 1 mole of 2-mercaptoethanol was added and reaction mixtures kept at 0°C following addition of enzyme preparation at zero time.

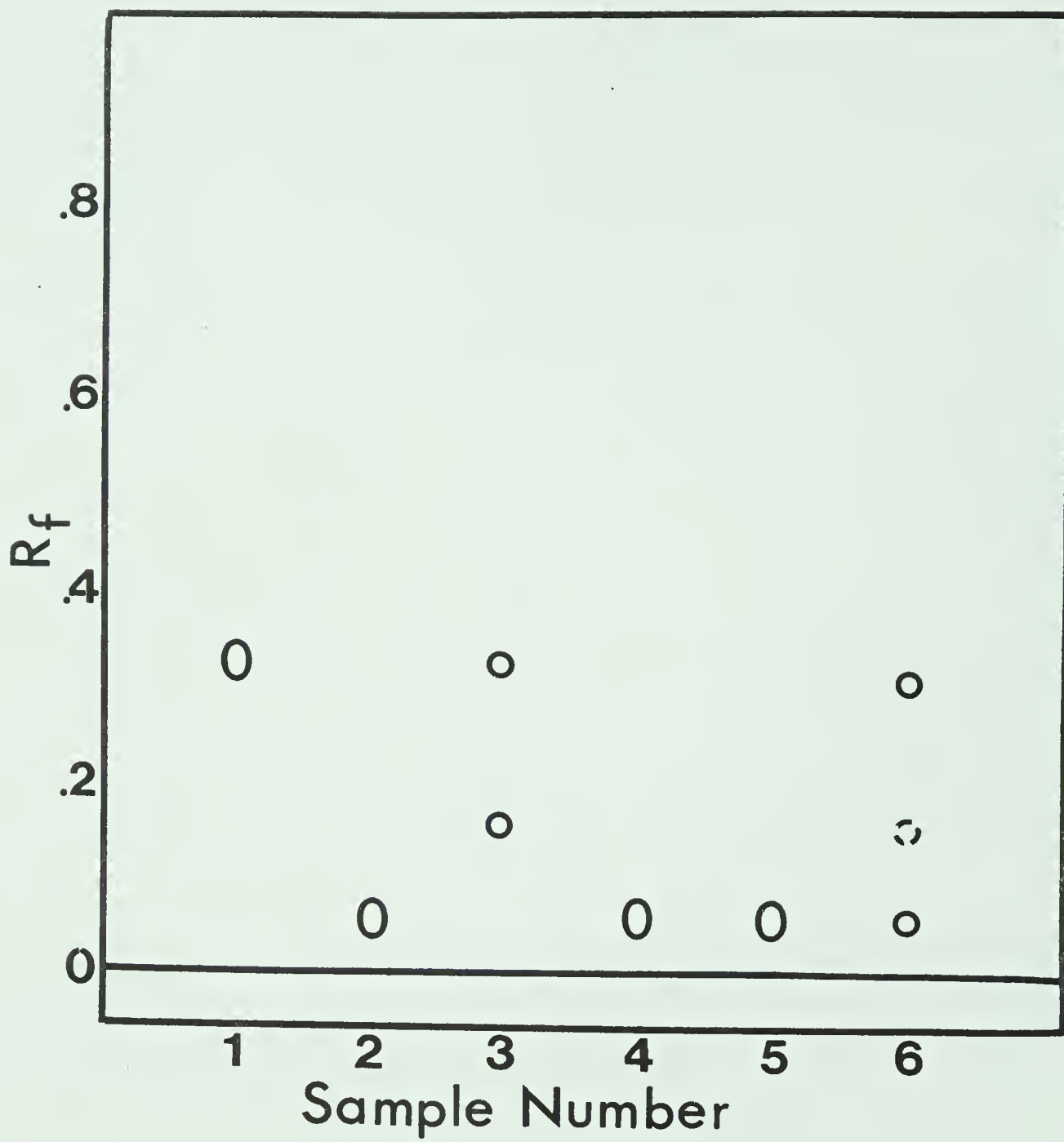


FIGURE 7

CHROMATOGRAPHIC IDENTIFICATION OF PRODUCTS
OF ENZYMATIC FOLATE SYNTHESIS

Enzymatic assay of folate products was carried out according to the method described in Materials and Methods. 5 mg/ml. protein was used. Twenty (20) ul. of reaction mixture was spotted on ascorbate-pres soaked Whatman No. 1 paper and chromatographed in 0.1 M phosphate buffer containing 6 mg/ml. ascorbate. Details of separation and bioautography are outlined in Materials and Methods.

LEGEND:

1. folate - 2.5 ng.
2. pteroate - 12.5 ng.
3. tetrahydrofolate - 12.5 ng.
4. complete system (ρ AB + glutamate)
5. reaction mixture minus glutamate
6. complete system (ρ ABG)

0.05 respectively as compared to values of 0.45 and 0.02 respectively as reported by Brown et al (1961).

With tetrahydrofolate (Sigma Co.) two spots were observed on the bioautogram, neither of which had the R_f value reported by Brown et al (1961) for tetrahydrofolate. The R_f of one of the spots was identical with that of folic acid; the R_f value of the second spot corresponded closely to the reported R_f value for dihydrofolate. It was quite possible that the commercial preparation was a mixture of oxidized compounds, namely folate and dihydrofolate or that the tetrahydrofolate had become oxidized during the course of chromatographic separation. Since authentic dihydrofolate was not available for the experiment, this aspect could not be confirmed.

Regarding the identity of products of the enzyme reaction in a complete system (ρ AB + glutamate), one spot corresponding to that of pteroate was noted; only one spot corresponding to that of pteroate was also demonstrated when glutamic acid was omitted from the reaction. Any attempt to demonstrate the formation of dihydrofolate as expected in a complete system according to the proposed biosynthetic pathway (Brown et al., 1961) was without success. The solvent system used did not allow the separation of dihydropteroate and pteroate.

When ρ AB and glutamate were replaced by ρ ABG, three spots were identified, two of which corresponded to folate and pteroate. The third one, although only faintly detectable on the bioautogram, had an R_f value identical to one of the spots

of the tetrahydrofolate standard; that spot was the one with the R_f value similar to that reported for dihydrofolate by Brown et al. (1961).

(c) Distribution of enzyme activity among different ammonium sulfate fractions. Although purification of the folate-synthesizing enzyme(s) was not attempted in the present study optimum conditions for ammonium sulfate fractionation were examined in order to obtain an enzyme preparation of highest activity. As illustrated in Table V, most of the protein was precipitated in the 20-40% and 40-60% fractionations. Highest specific activities were demonstrated in these fractions also. Consequently, all enzyme preparations were obtained from 20-60% fractionations that were dialysed before use. After dialysis, enzyme preparations stored at 4°C were found to retain more than 60% of their original activities after one month.

(d) Optimum conditions for enzyme assay. Varying the amount of protein in the reaction mixture indicated a linear relationship between protein concentration and enzyme activity at protein concentrations ranging from 2.0 to 7.0 mg./ml. (Fig 8). Protein concentrations of 2.0 to 4.0 mg./ml. were used routinely in enzyme assays.

Optimum concentrations for DHP (Fig. 9A) and ρ AB (Fig. 9B) were found to be 40 and 50 μ M respectively. With higher concentrations of DHP, the enzyme reaction was somewhat inhibited. No pH effect could be observed to account for such inhibition. The requirement for ATP and Mg^{2+} was

TABLE V

Protein Yield and Enzyme Activity of Folate-Synthesizing Enzymes
as a Function of Ammonium Sulfate Fractionation

% (NH ₄) ₂ SO ₄ Fractionation	Protein		Volume	Total		Specific		Total
	Conc'n	(mg/ml)	(ml)	Protein	(mg)	Activity	Activity	Activity
		(mg/ml)						
0-20%	6		0.4	2.4		<0.5x10 ⁻³		<1.2x10 ⁻³
20-40%	35		2.1	74		1.4x10 ⁻³		104x10 ⁻³
40-60%	42		2.0	84		1.8x10 ⁻³		151x10 ⁻³
60-90%	19		2.4	46		<0.5x10 ⁻³		< 23x10 ⁻³

a Specific activity is defined as nmoles of folate equivalents formed per minute per mg. protein.

E. coli K-12-AB-301 was grown in Davis-Mingioli medium with added casamino acids for 12 hours on a gyrotoryshaker (200 rpm) at 37°C. Cells were harvested, sonicated and treated with protamine sulfate as indicated in Materials and Methods.

Ammonium sulfate fractionations were carried out according to procedure outlined in Materials and Methods. Enzymatic assay of the above fractions was set up as set forth in Materials and Methods. A final protein concentration of 5 mg/ml was used in the assay.

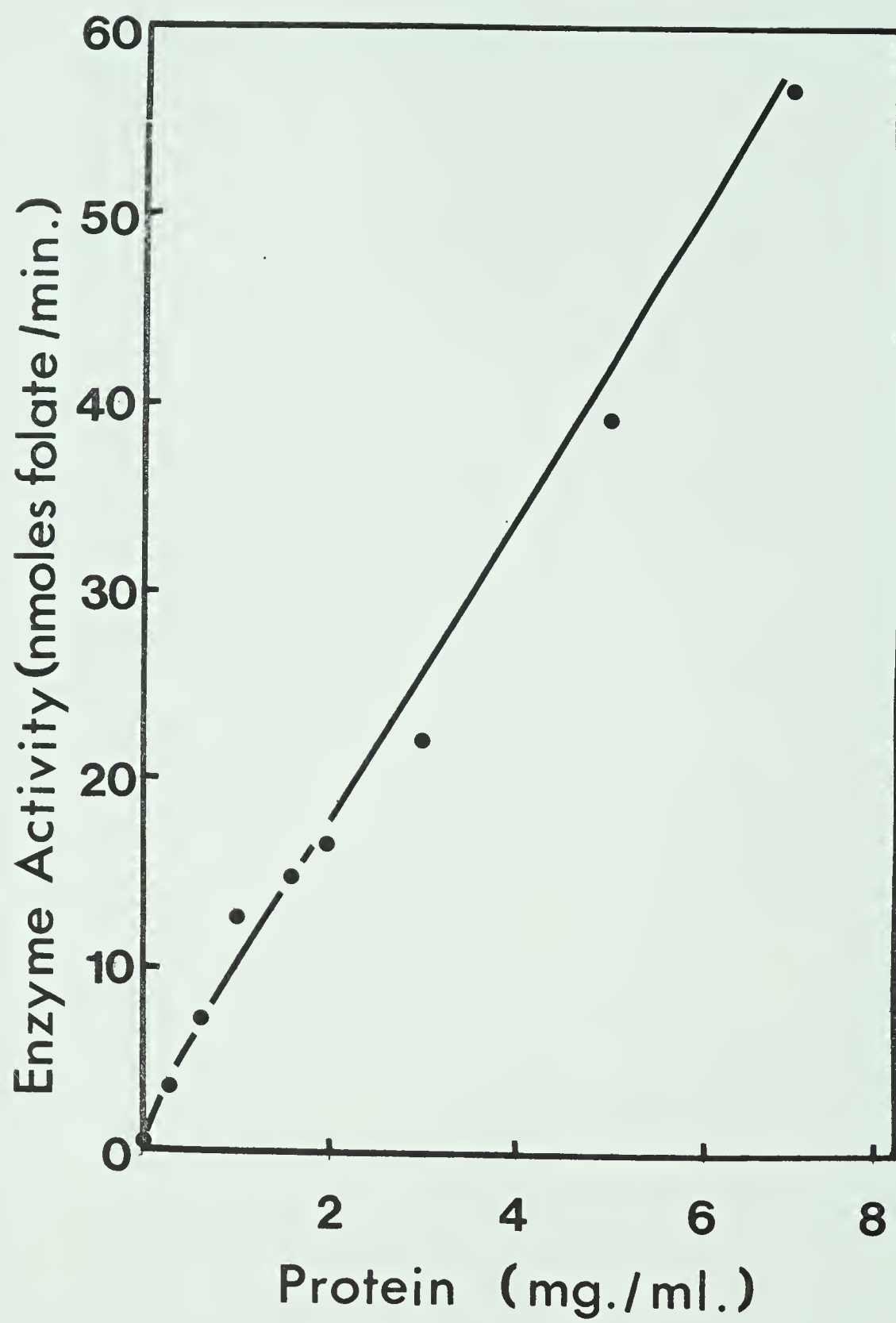


FIGURE 8

EFFECT OF PROTEIN CONCENTRATION ON THE ACTIVITY OF
THE FOLATE-SYNTHESIZING ENZYME SYSTEM

E. coli K-12-AB-301 was grown according to procedure outlined in Table V. The protein fraction was prepared from a 20-60% ammonium sulfate fractionation as outlined in Materials and Methods.

Various amounts of protein were added to the enzyme assay reaction mixtures at time zero. Enzyme assay conditions were as described in Materials and Methods.

Enzyme activity is defined as nanomoles of folate equivalents formed per minute per total protein present in the system.

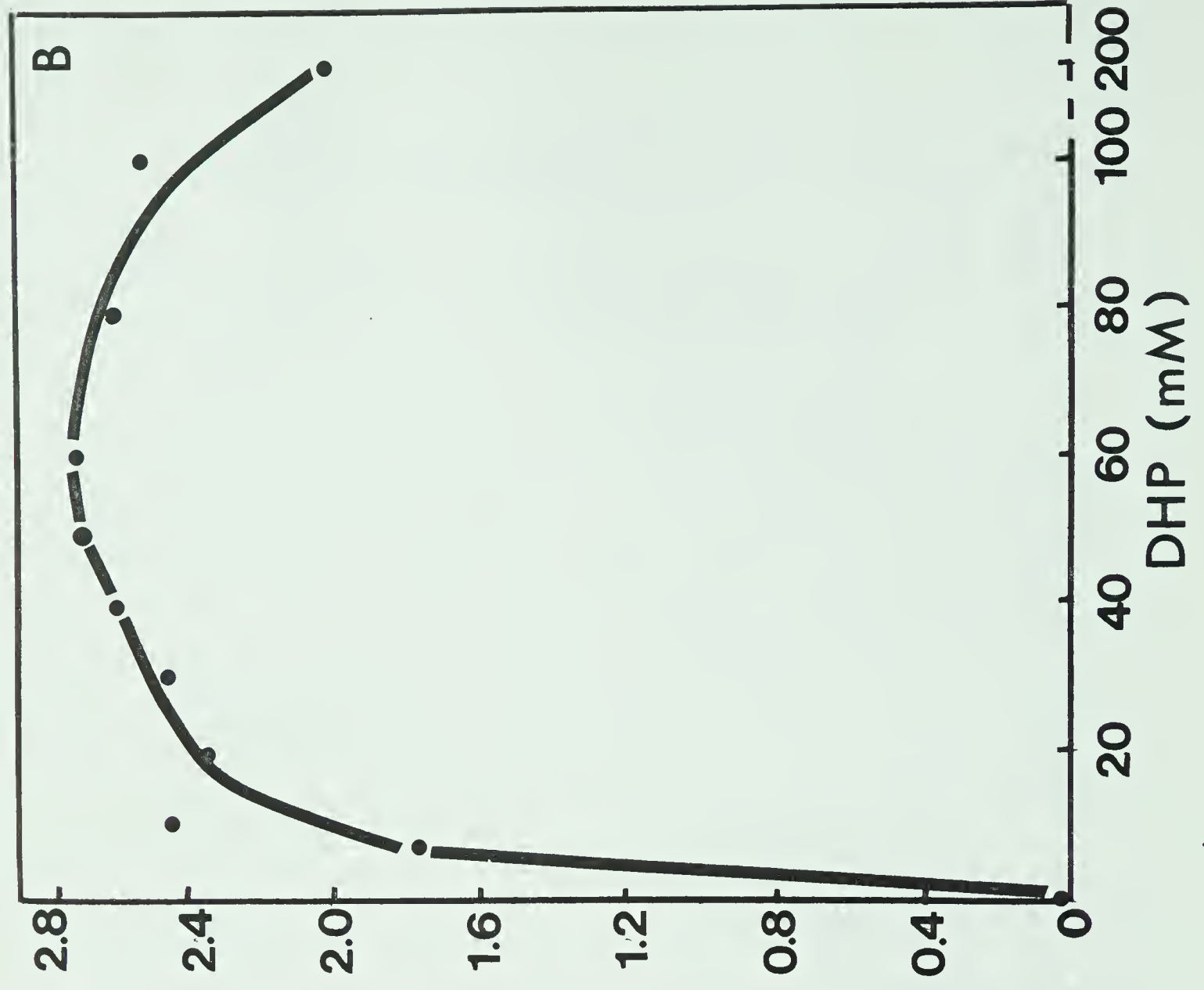
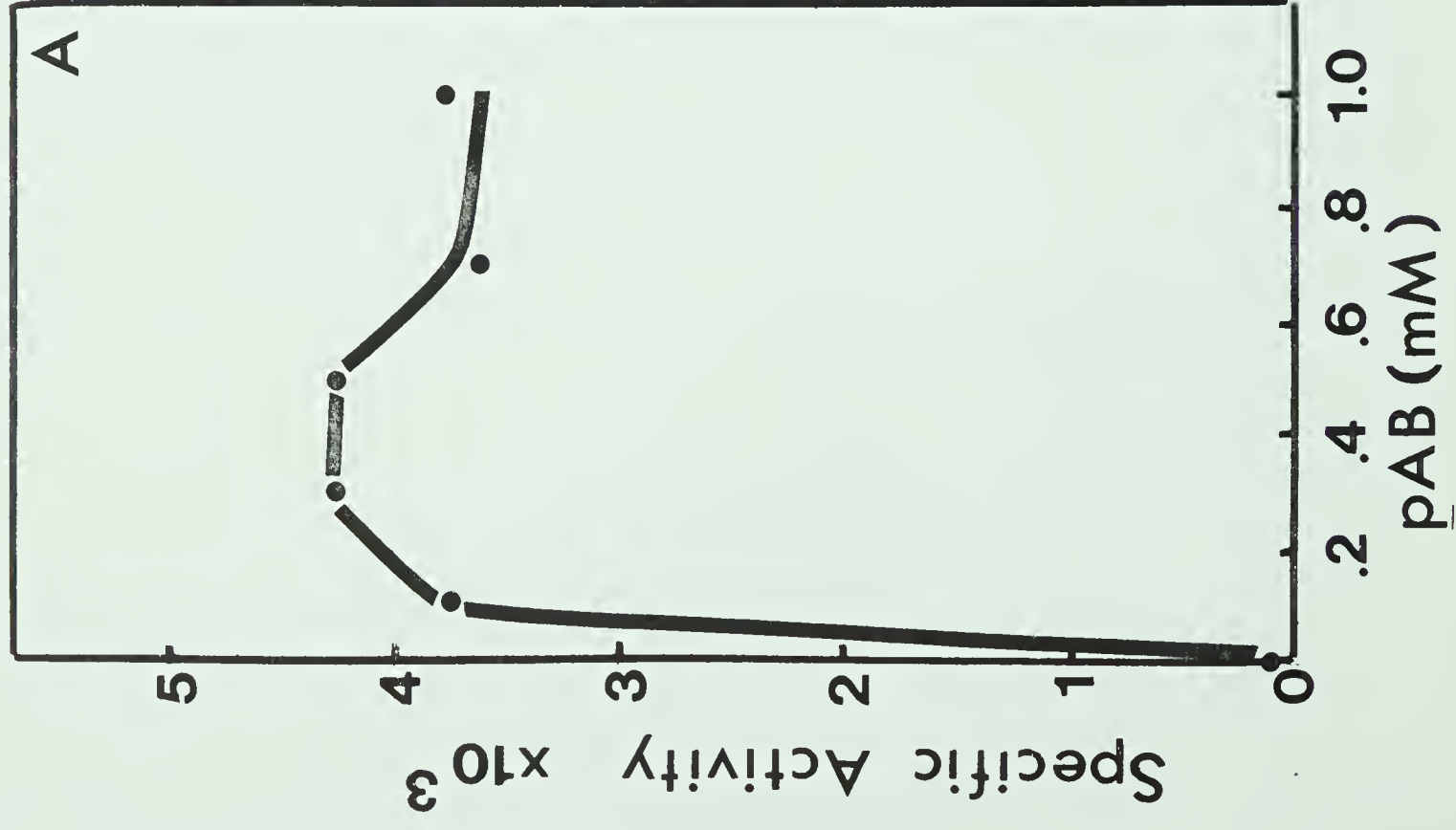


FIGURE 9

EFFECT OF VARYING SUBSTRATE CONCENTRATIONS ON THE SPECIFIC
ACTIVITY OF THE ENZYME SYSTEM

A: p - aminobenzoic acid

B: 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine

Enzyme assay conditions were as described in
Materials and Methods. Protein Concentration used was
2 mg/ml. Specific activity is expressed as nmoles of
folate equivalent formed per mg. protein per minute.

satisfied at optimal concentration of 100 mM for ATP and 5.0 mM for MgCl_2 (Fig. 10 A&B).

The effect of pH and buffer molarity was examined (Fig. 11 A&B). A pH range of 7.8-8.0 was found to be optimum although a relatively broad pH spectrum was shown in the results. Brown and coworkers (1961) used pH 7.0 in their studies while other workers (Shiota et al, 1963; Wolf and Hotchkiss, 1963) used pH 8.0. The molarity of the buffer (Tris-HCl) appeared to have little effect on enzyme activity.

The optimum temperature for incubation was found to be 37°C (Fig. 12). However, because the rate of reaction was linear at 30°C over a wide range of incubation times while that at 37°C was not linear, all enzyme assays were performed at 30°C. (Fig. 13).

In the system of Brown and his coworkers, the enzyme assay was always incubated for 3 hours under anaerobic conditions. In a recent paper by Ortiz (1970) it was suggested that incubation be carried out under aerobic conditions for a shorter period of time (30 min.). In the present study, a comparison of aerobic and anaerobic incubation conditions for varying periods of time was made. Anaerobic conditions were achieved by placing reaction mixtures (1.0 ml.) in Thunburg tubes, evacuating the tubes with an aspirator for 2 min. and flushing tubes for 45 sec. with hydrogen. The procedure was repeated twice before reaction mixtures were incubated. As shown in Fig. 14, anaerobic conditions did not appear to enhance the specific activity of the enzyme system. Inclusion

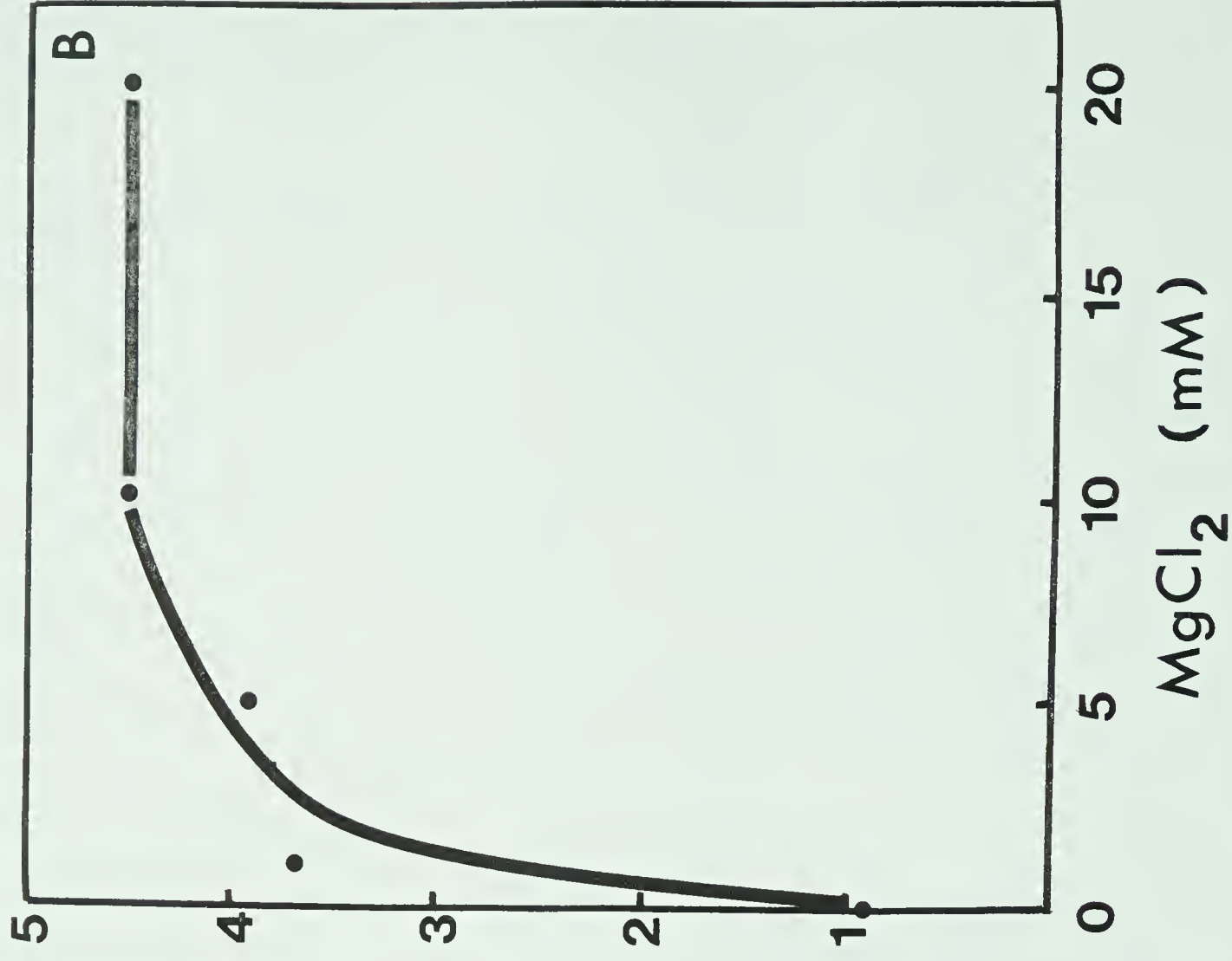
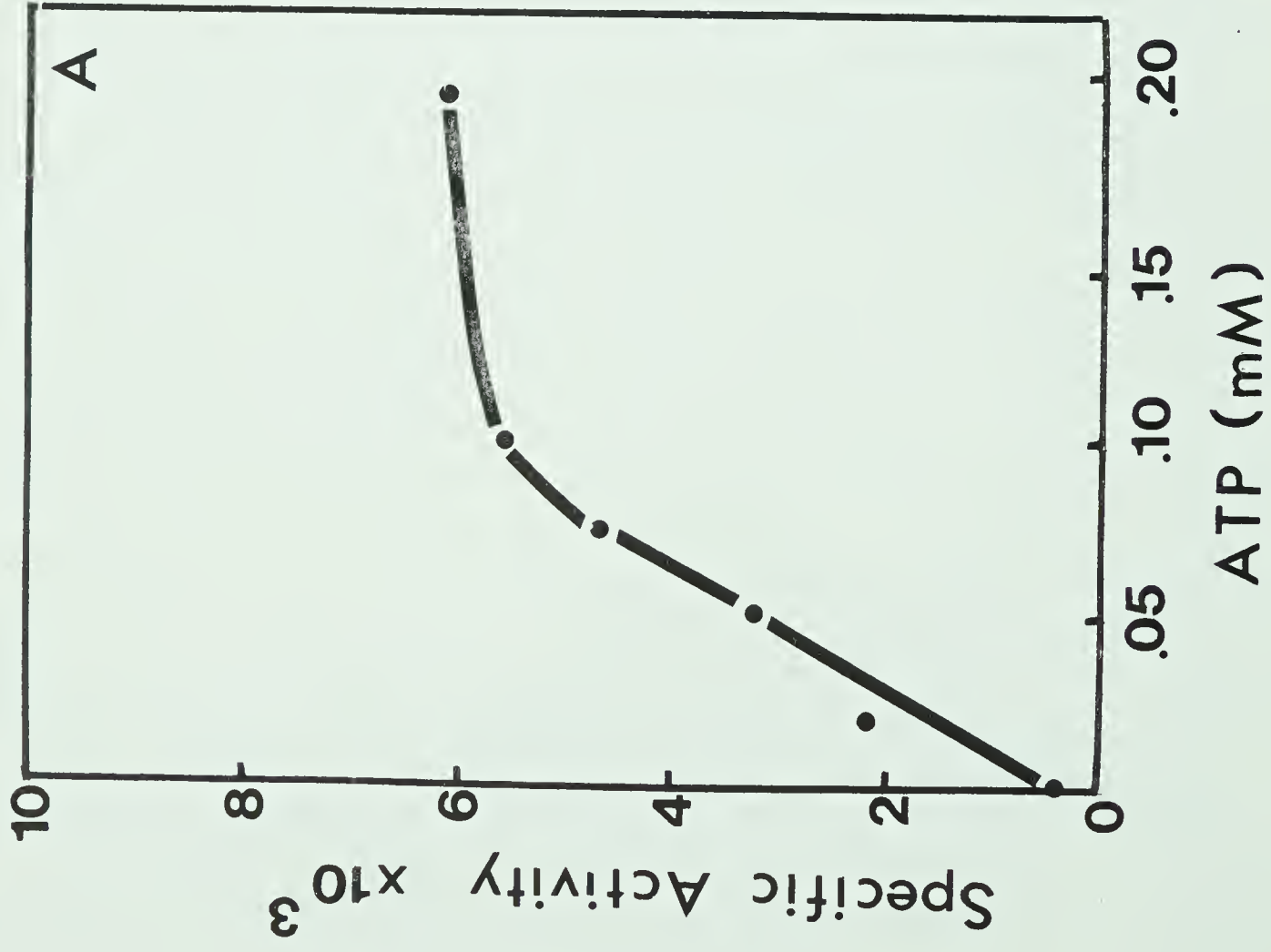


FIGURE 10

OPTIMAL COFACTOR REQUIREMENTS FOR THE FOLATE ENZYME SYSTEM

A: ATP

B: MgCl_2

Enzyme conditions were as described in Materials and Methods. Protein concentration was 2 mg/ml.

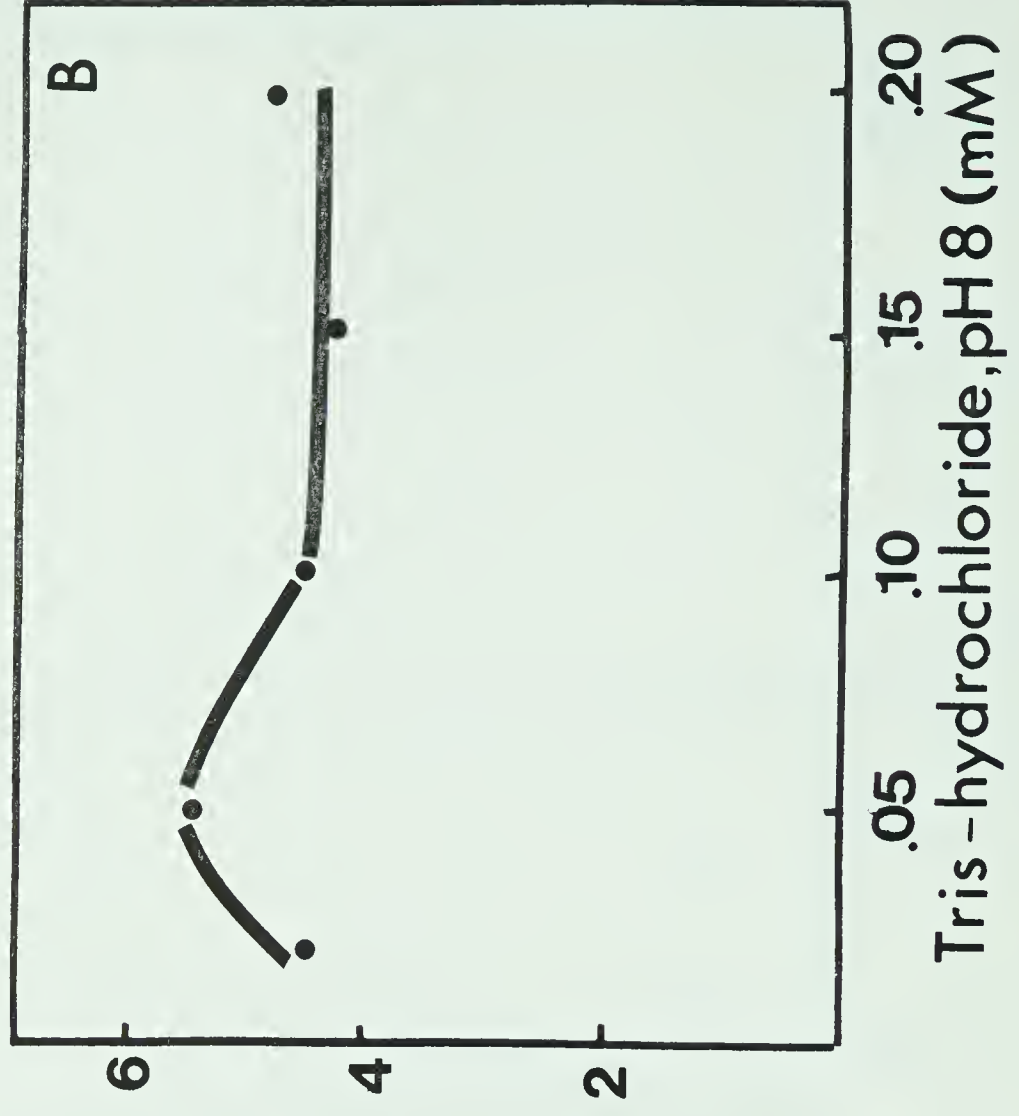
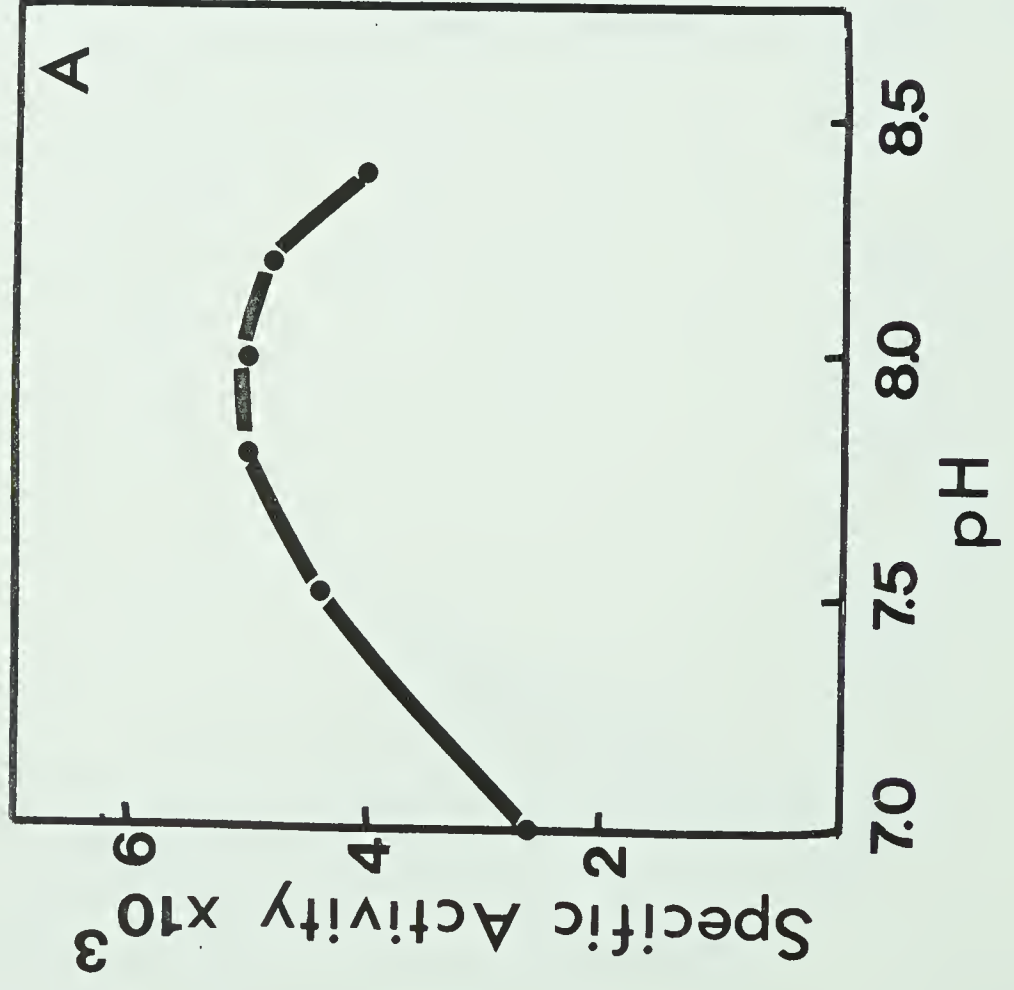


FIGURE 11

EFFECT OF BUFFER CONDITIONS ON THE FOLATE ENZYME SYSTEM

A: pH

B: Tris-hydrochloride molarity

Enzyme assay conditions were as described in Materials and Methods. Protein concentration used was 2 mg/ml. The pH of Tris buffer used in (b) was 8.2.

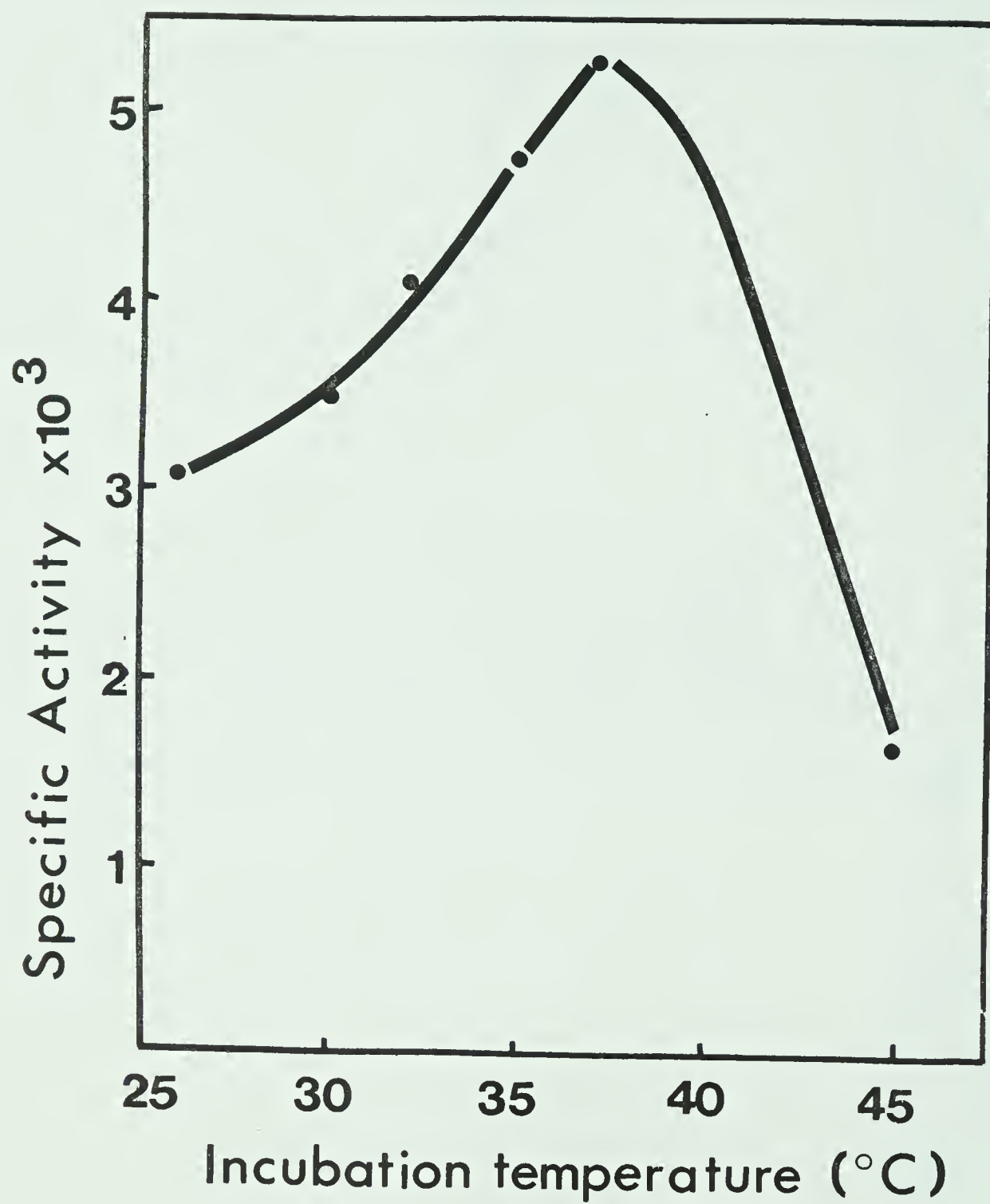


FIGURE 12

EFFECT OF INCUBATION TEMPERATURE ON THE SPECIFIC ACTIVITY
OF THE FOLATE SYSTEM

Enzyme assay conditions were as described in Materials and Methods. Incubation time was 120 min. Protein concentration was 2 mg/ml.

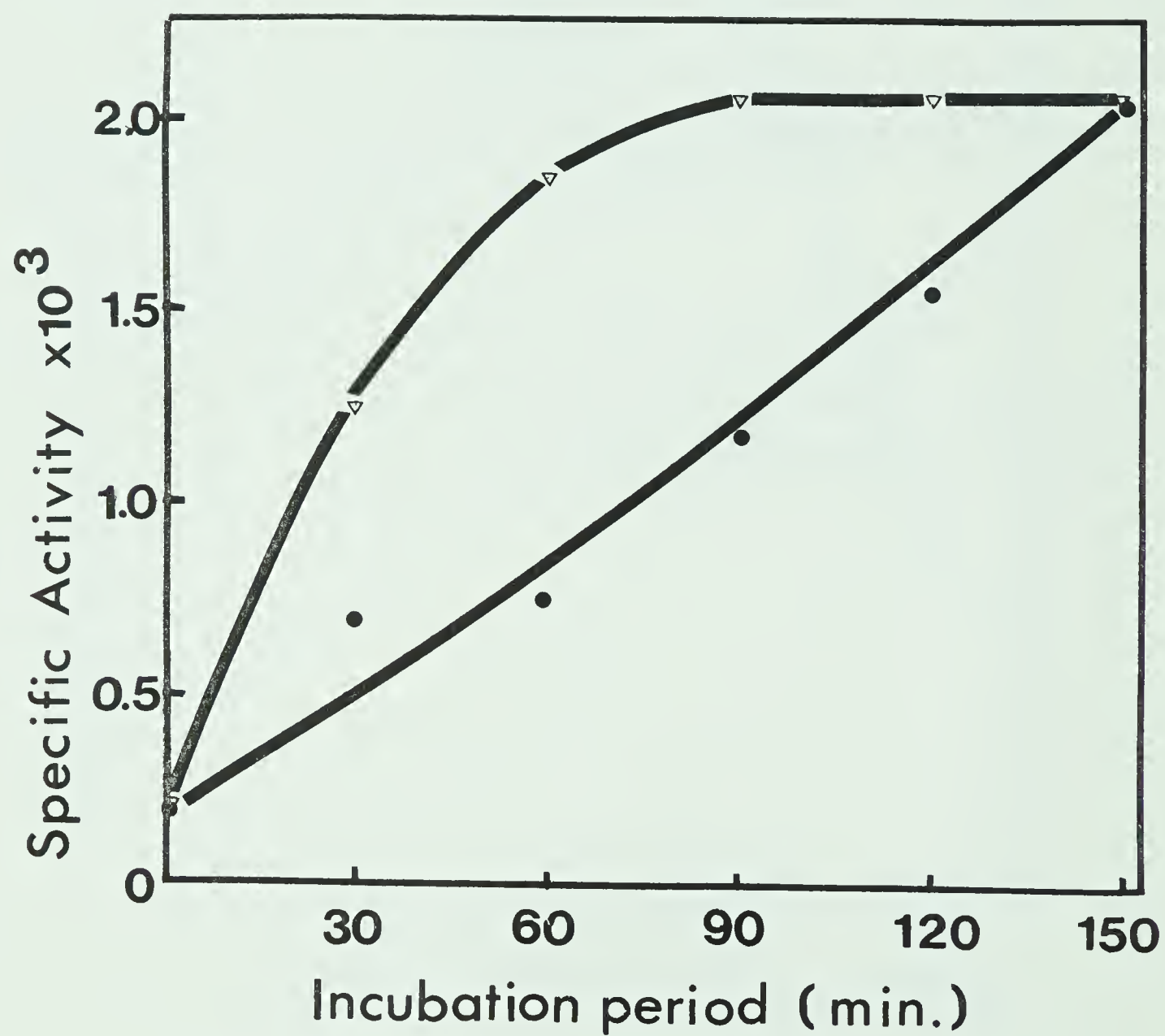


FIGURE 13

EFFECT OF INCUBATION TIME AT DIFFERENT TEMPERATURES
ON THE SPECIFIC ACTIVITY OF THE FOLATE
ENZYME SYSTEM

Enzyme assay conditions were as described in Materials and Methods and carried out aerobically as outlined in Fig. 14.

LEGEND:

● — ● - 30°C

▽ — ▽ - 37°C

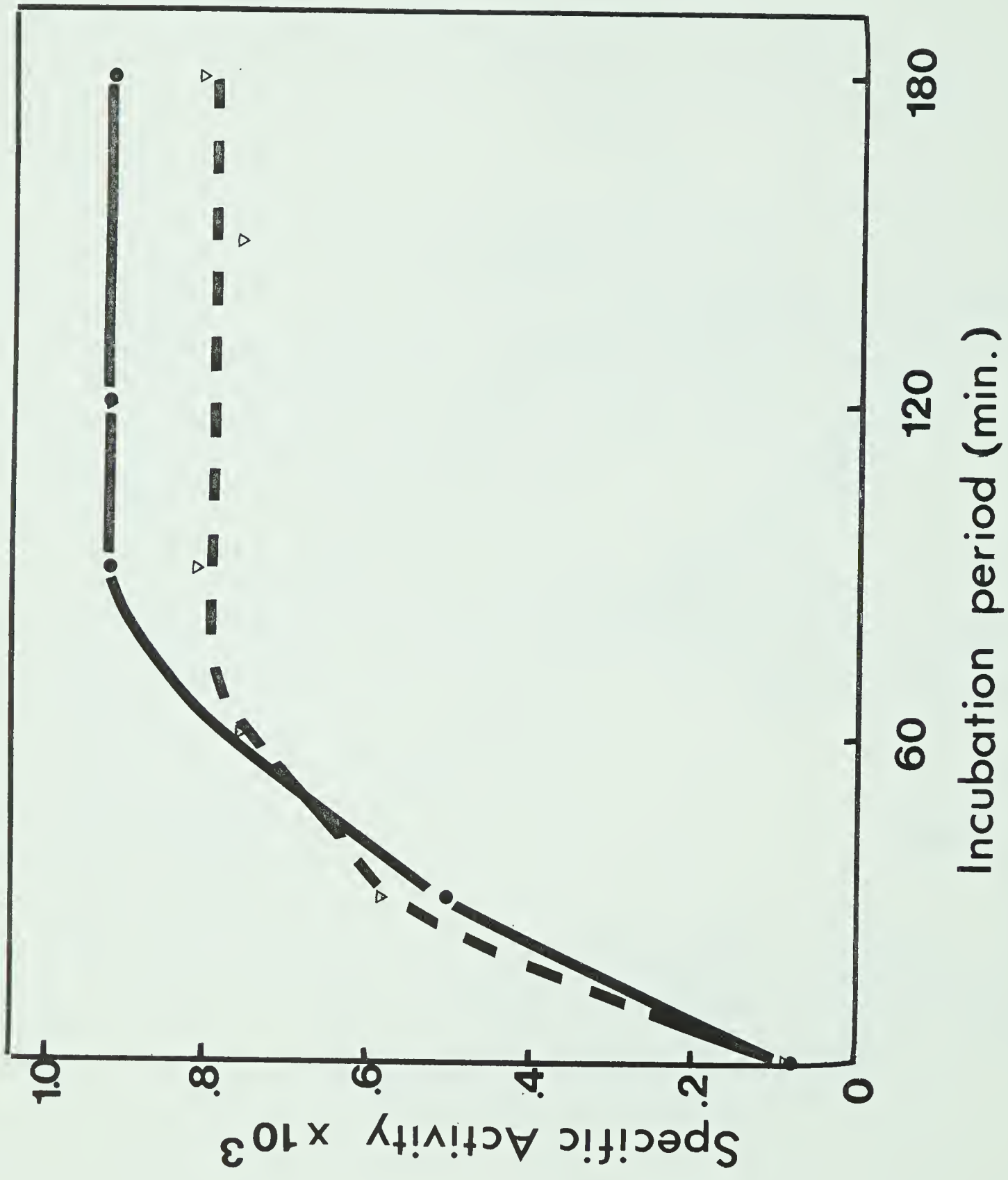


FIGURE 14

EFFECT OF AEROBIC AND ANEROBIC INCUBATION ON THE
ACTIVITY OF THE FOLATE ENZYME SYSTEM

Enzyme assay conditions were as described in Materials and Methods. The anaerobic incubation was carried out as outlined in the text. Aerobic incubation was done in 200 ul. volumes in micro-assay tubes in a Beckman 154B tempbloc. Reaction was stopped at various time intervals.

LEGEND:

●—● - aerobic

▽—▽ - anaerobic

of 67 mM 2-mercaptoethanol in reaction mixtures appeared to be sufficient to keep the system in a reduced state for the duration of the incubation period. Because of the relative simplicity of the aerobic incubation as compared to anaerobic incubation, all studies were performed under aerobic conditions using a 90 min. incubation.

The method used for stopping the reaction was examined also. Addition of 10% trichloroacetic acid to stop the reaction resulted in a complete inactivation or destruction of reaction products. No pH change in S. faecalis assay medium upon addition of assay amounts of TCA-treated reaction mixture was observed that might account for it. Addition of mercaptoethanol or cooling of reaction mixture in an ice bath was found satisfactory in stopping the reaction. In the aerobic system reactions were stopped routinely by placing assay tubes in a dry ice-acetone bath for 5 sec. and then the samples were stored at 4°C until used for microbiological assay.

BIOCHEMICAL CHARACTERIZATION OF
SULFONAMIDE-RESISTANT STRAINS

Effect of Sulfonamides on the Synthesis
of Folic Acid in Cell-Free Systems

The effect of sulfonamides on enzyme activity was investigated in cell-free systems of both sensitive and resistant strains of E. coli K-12-AB-301. As illustrated in Fig. 15, the synthesis of folate in the cell-free extract of the sensitive strain is inhibited by increasing concentrations of sulfadiazine. A 50% inhibition of the enzyme reaction was obtained when the sulfadiazine concentration was 0.1 mM; this was about twenty-five times greater than the 50% limiting sulfonamide level for growth of the same organism. (Tables 3 and 6).

The effect of sulfadiazine on the synthesis of folic acid in cell-free systems of sensitive and resistant strains was compared; the results, expressed in terms of % enzyme activity, are shown in Fig. 16. The folate-synthesizing systems of both sensitive and resistant strains were inhibited equally in the presence of increasing concentrations of sulfadiazine. With a concentration of sulfadiazine equal to 100 μ M, enzyme activity was reduced to one-half of that in the absence of sulfadiazine. Similar results were obtained using the E. coli K-12-58 cell-free systems (Table 6).

TABLE VI

Effect of Sulfonamides on the Enzyme Activity
of Cell-Free Extracts of E. coli

Strain	^a 50% Limiting Sulfonamide Level			
	Sulfadiazine	Sulfamerazine	Sulfathiazole	Sulfamethazine
<u>E. coli</u> K-12-AB-301	0.09-0.11 mM	0.09-0.12 mM	0.03-0.05 mM	0.04-0.05 mM
" K-12-AB-301-5	0.08-0.10 mM	0.08-0.11 mM	0.01-0.03 mM	0.05-0.11 mM
" K-12-AB-301/222	0.07-0.09 mM	0.06-0.08 mM	0.01-0.02 mM	0.05-0.07 mM
<u>E. coli</u> K-12-58	0.09-0.11 mM	-	-	-
" K-12-58-3	0.08-0.10 mM	-	-	-
" K-12-58/222	0.11-0.14 mM	-	-	-

^a50% limiting sulfonamide level - the concentration of sulfonamide required to limit the specific activity of the cell-free extract to one-half that of the cell-free extract in the absence of sulfonamide.
A 20-60% ammonium sulfate fraction of each of the systems was used. Details of the procedures are found in Materials and Methods.

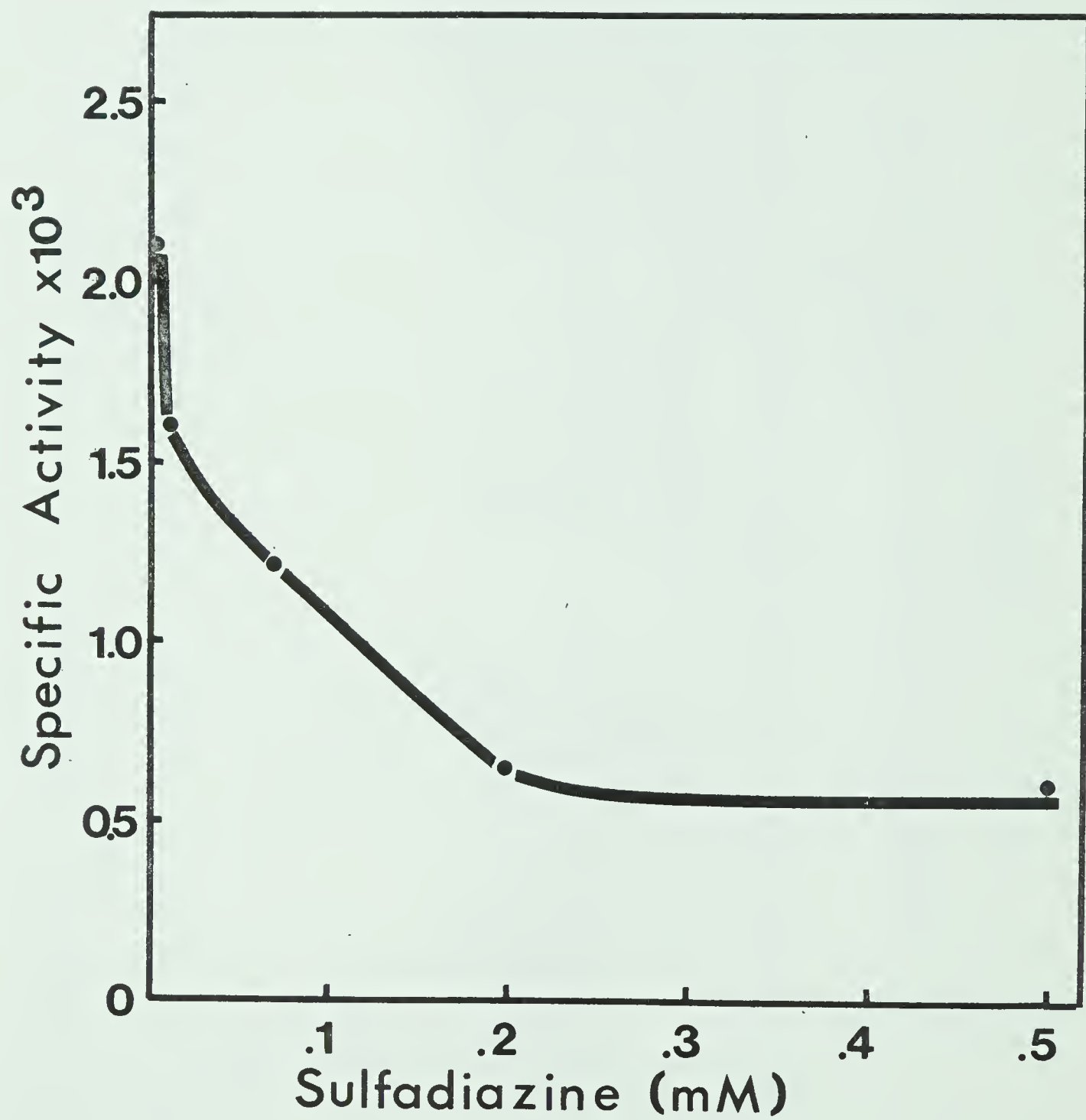


FIGURE 15

Effect of Sulfadiazine on Folate Synthesis by
Cell-Free Extracts of a Sulfonamide-sensitive Strain,
E. coli K-12-AB-301.

The enzyme assay was carried out under conditions described in Materials and Methods. Two (2) mg/ml. of protein were used. Various concentrations of sulfadiazine were added to the reaction mixtures at time zero. Formation of folates was measured by microbiological assay using S. faecalis 8043.

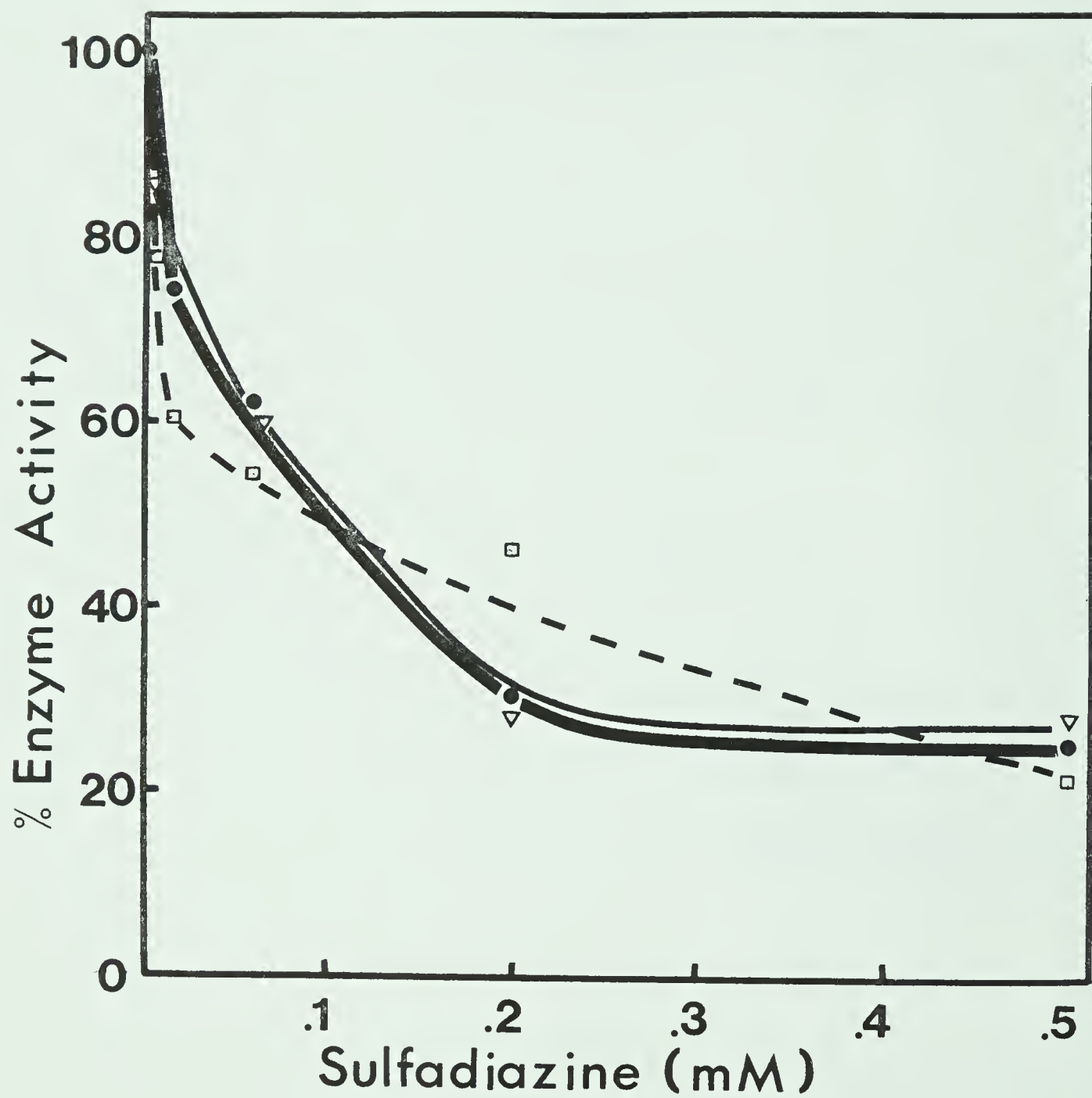


FIGURE 16

INHIBITION OF FOLATE SYNTHESIS IN CELL-FREE EXTRACTS
OF E. COLI BY SULFADIAZINE

Assay conditions are identical to those for
Fig. 15.

LEGEND:

- - E. coli K-12-AB-301
- ▽—▽ - E. coli K-12-AB-301-5
- - E. coli K-12-AB-301/222

Because of the differences in 50% limiting sulfonamide levels when different sulfonamides were used in the growth studies, the various sulfonamides were also tested in cell-free systems (Table VI). For each of the sulfonamides investigated, sensitive and resistant strains showed similar 50% limiting sulfonamide levels. However, as with whole cell systems, the cell-free systems were more sensitive to the action of sulfathiazole and sulfamethazine than to sulfadiazine and sulfamerazine; a 2-3 fold difference in sensitivity levels was consistent with a difference of up to 5-fold for the whole cell systems.

Kinetic Studies of the Folate-Synthesizing Enzyme Systems

Because initial studies on the cell-free enzyme systems revealed 20-fold higher enzyme activities in resistant strains than in sensitive strains, there existed a possibility of different enzyme systems being involved in folate biosynthesis; however, this difference has since been questioned.

Michaelis-Menton constants were determined for p-aminobenzoic acid in sensitive and resistant strains (Table VII). $K_m(\underline{p}AB)$ values ranging from 1.2 to 4.2×10^{-6} M were obtained in all systems; with regard to $K_m(\underline{p}AB)$ then, there appears to be no significant difference between the enzymes.

The determination of K_i values in the presence of 0.2×10^{-6} M sulfadiazine for the sensitive strain, and 40×10^{-6} M sulfadiazine for the resistant strains was without success. The cell-free system consists of a multi-enzyme

TABLE VII

Kinetic Analysis of the Folate-Synthesizing Enzyme System
of E. coli K-12-AB-301

<u>E. coli</u> Strain	^a V _{max} (nmoles/min/mg)	^b K _m (ρAB) (10 ⁻⁶ M)
K-12-AB-301	0.02	1.2 - 1.3
K-12-AB-301-5	0.02 - 0.04	1.3 - 4.0
K-12-AB-301/222	0.03 - 0.05	2.9 - 4.2

The enzyme assay was carried out as described in Materials and Methods using ρAB concentrations ranging from 0 to 5 μM. A protein concentration of 2 mg/ml was used. Products of the reaction were assayed using S. faecalis. Results were plotted in a Lineweaver-Burke format of 1/v vs. 1/s.

^aV_{max} is defined as the theoretical limiting velocity (nmoles/min/mg) at infinite substrate concentration.

^bK_m is defined as a constant characteristic of the enzyme and the reaction system.

complex which may have accounted for the inconsistencies when Lineweaver-Burke analyses were attempted. Kinetic studies were not further pursued.

Levels of Folate-Synthesizing Enzymes During Growth

The amount of folate-synthesizing enzyme present in sensitive and resistant strains during the growth cycle of each was investigated. When the cells had reached the stationary phase (~ 5 hr. growth), the specific activity of the resistant strains as compared to the sensitive strain was approximately 2-fold higher (Table VIII). The levels of the folate-synthesizing enzyme were examined in more detail by taking samples of the cultures at different incubation periods in the presence and absence of sulfadiazine; the results are shown in Fig. 17. Approximately a 2-fold difference in the specific activities of the two enzyme systems existed throughout the growth curve; subinhibitory concentrations of sulfadiazine did not appear to affect the enzyme activity of either strain at any stage of growth (Fig. 17).

Folate Synthesis *in vivo*

As a consequence of the 2-fold difference in specific activity of the folate-synthesizing enzymes in sensitive and resistant strains of *E. coli* K-12-AB-301, an investigation of the folate synthesis *in vivo* was made. Both intra- and extra-cellular forms of folate were analysed. The total amount of folate synthesized *in vivo* in the absence of sulfonamides was

TABLE VIII

Comparison of Specific Activities of the Folate-Enzyme System
in Sensitive and Resistant Strains of E. coli K-12-AB-301

Strain	S. A. (nmoles/min/mg) x 10 ³
K-12-AB-301	9.5
K-12-AB-301-5	18.0
K-12-AB-301/222	20.0

After 5 hours growth (stationary phase), cells were harvested, washed and sonicated. Cell-free enzyme preparations were made according to Materials and Methods.

Enzyme activity was determined using the complete reaction mixture described in Materials and Methods.

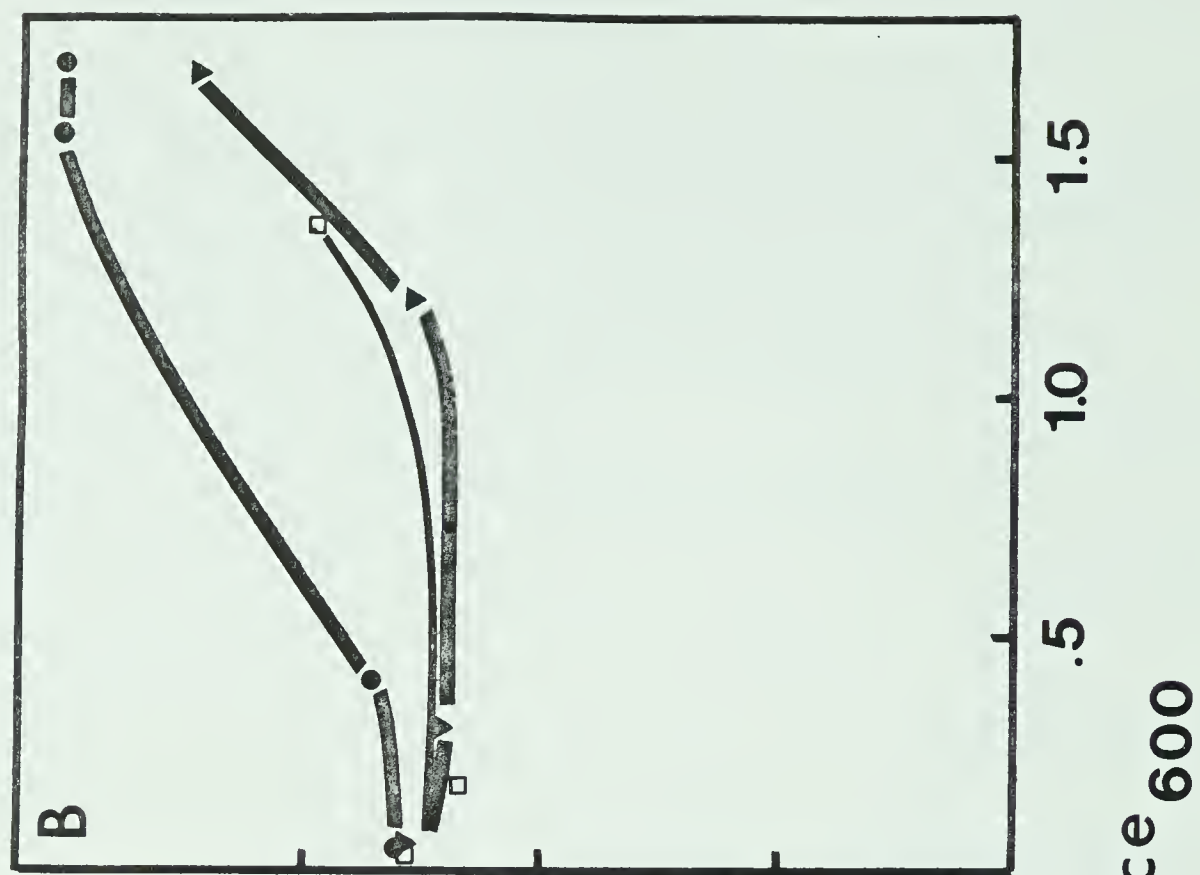
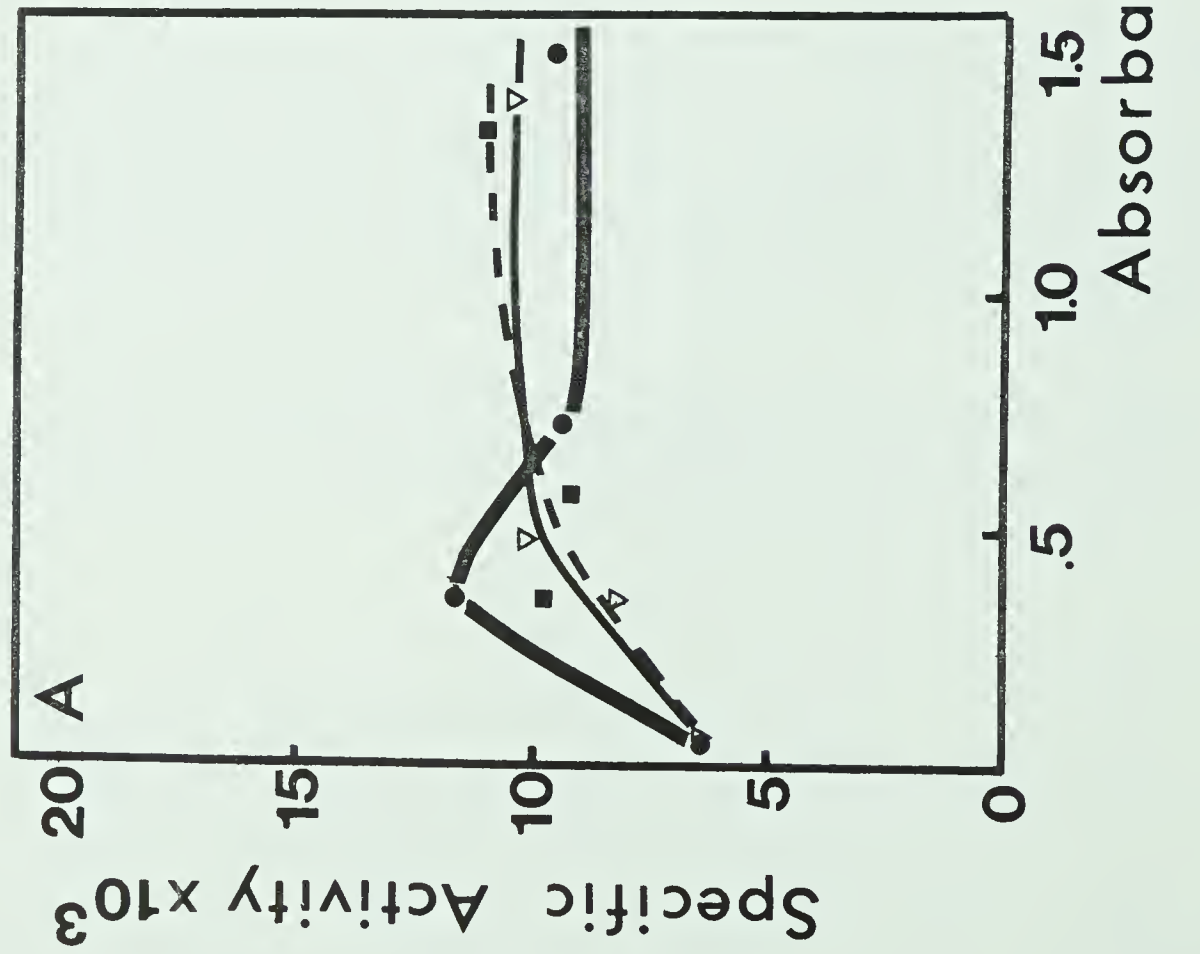


FIGURE 17

A. Effect of sulfadiazine on the level of folate-synthesizing enzymes during growth of the sensitive strain, E. coli K-12-AB-301.

B. Effect of sulfadiazine on the level of folate-synthesizing enzymes during growth of the resistant strain, E. coli K-12-AB-301/222.

LEGEND:

A. ●—● 0 ug/ml Sd
 ▼—▼ 0.5 ug/ml Sd
 ■—■ 1.0 ug/ml Sd
 B. ●—● 0 ug/ml Sd
 ▼—▼ 250 ug/ml Sd
 □—□ 500 ug/ml Sd

Overnite cultures of K-12-AB-301 and K-12-AB-301/222 were inoculated in triplicate into free Davis-Mingioli medium containing 50 mg/l. methionine to an initial absorbance of 0.03. For the sensitive strain, final sulfadiazine concentrations of 0, 0.5 and 1.0 ug/ml. were added; for the resistant strain, Sd concentrations of 0, 250 and 500 ug/ml. were added. Cultures were grown in 1-liter volumes in 2-l. flasks at 37°C on an N.B. Scientific Gyrotory shaker. At points along the growth curve, 1-liter aliquots were removed, cells harvested and sonicated and the enzyme system purified according to Materials and Methods. Enzymatic and microbiological assay of the products was carried out.

compared in sensitive and resistant strains (Fig. 18). During logarithmic growth, the two resistant strains synthesized 2-3 times as much folate as did the sensitive strain. A sharp decline in folate synthesized per mg. dry weight of cells was observed at the onset of the stationary phase.

The presence of subinhibitory concentrations of sulfonamide during growth appeared to inhibit folate synthesis in both sensitive and resistant strains to almost one-half that in the absence of sulfonamides (Fig. 19). From Fig. 19 it may also be noted that the total folate synthesis by resistant strains in the presence of sulfonamides was similar to the total amount of folate synthesized by the sensitive strain in the absence of sulfonamide.

Extracellular folate levels were found to be slightly higher than intracellular folate levels. However when cells were grown in the presence of the same sulfonamide concentration, synthesis of both intra- and extra-cellular folate were affected to some extent (Fig. 20).

Total folate as analysed may include many different derivatives of folic acid. A comparison of types of folates assayable by microbiological means was presented in Table I. Total folate synthesis was assayed using both S. faecalis and L. casei. More folate was found to be assayable with L. casei than with S. faecalis suggesting the presence of methylated and polyglutamyl folate derivatives not assayable by the S. faecalis method (Fig. 21). The synthesis of L. casei assayable folate too was inhibited by the sulfonamide.

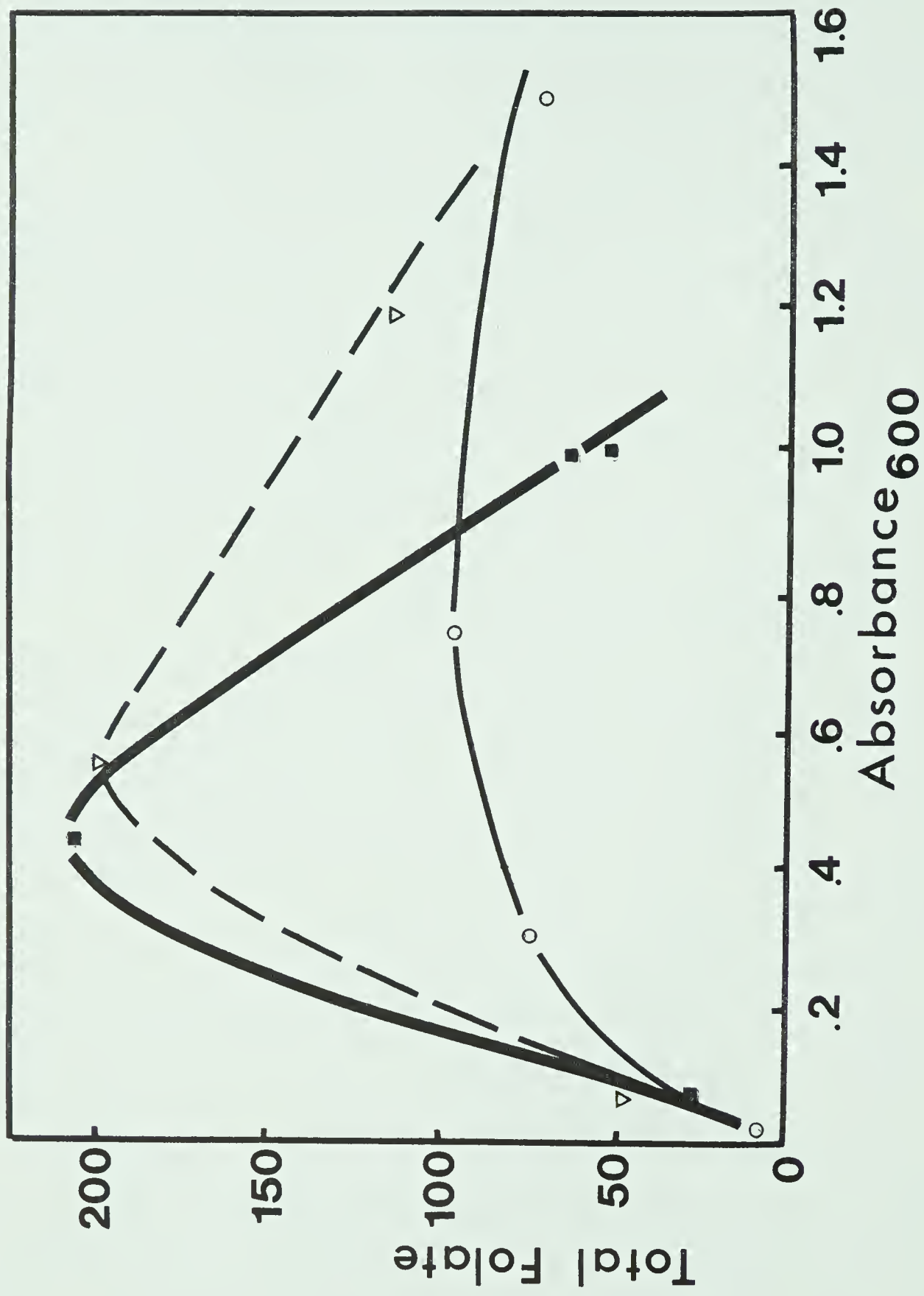


FIGURE 18

Amount of Total Folate Present in Cultures of Sensitive and Resistant Strains of E. coli K-12-AB-301 as a Function of Growth.

Cells were grown in the presence of 0, 0.5 or 1.0 ug/ml. sulfadiazine. Extracellular folate was removed from cultures for analysis by centrifugation and washing as described in Materials and Methods; intracellular folate was released from the cells by acid hydrolysis (see Materials and Methods). Folates were assayed microbiologically using S. faecalis 8043.

"Total folate" is defined as the sum of the intracellular and extracellular folate of a growing culture. It is expressed as nmoles folate per mg. dry wt. of cells.

LEGEND:

○—○ - E. coli K-12-AB-301

■—■ - E. coli K-12-AB-301-5

▽--▽ - E. coli K-12-AB-301/222

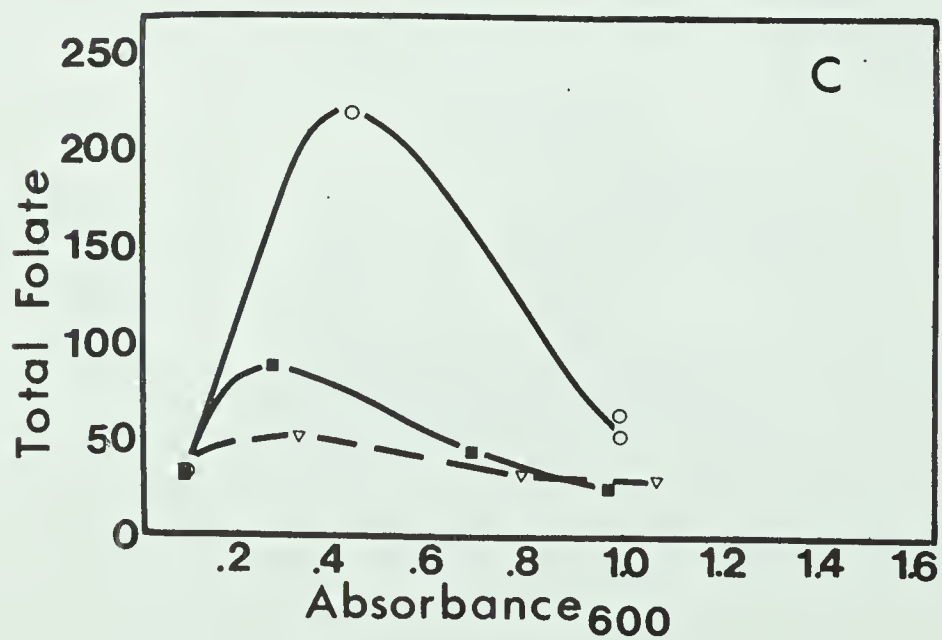
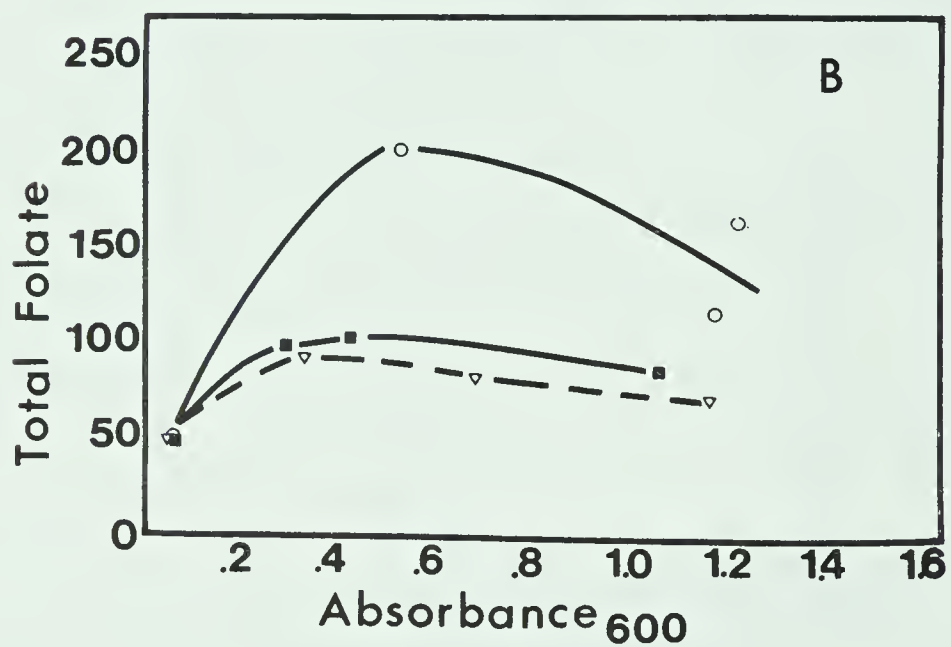
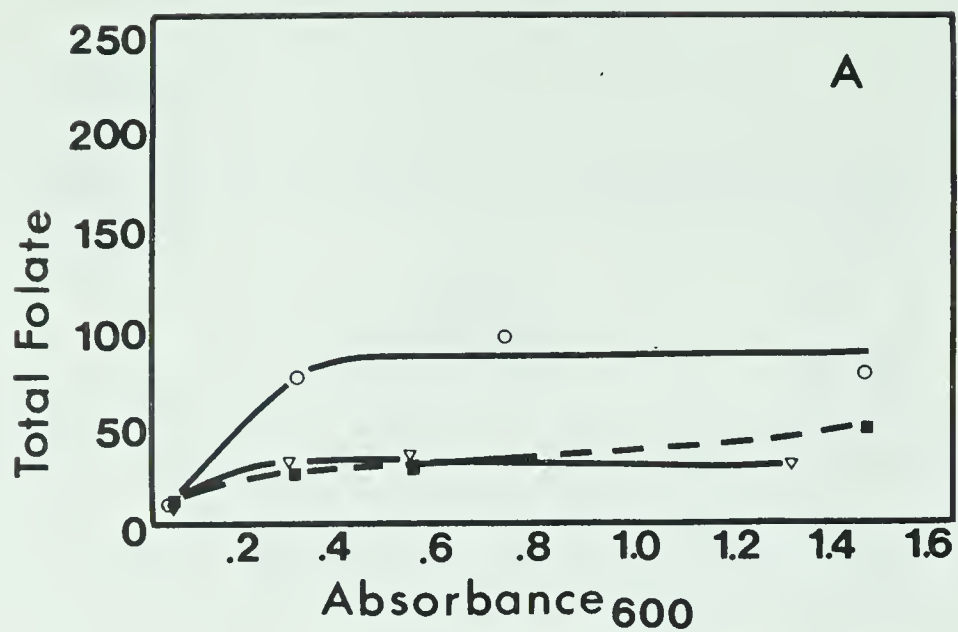


FIGURE 19

EFFECT OF SULFONAMIDE CONCENTRATION ON THE AMOUNT OF
TOTAL FOLATE PRESENT IN CULTURES OF SENSITIVE
AND RESISTANT STRAINS OF E. COLI K-12-AB-301
AS A FUNCTION OF GROWTH

Growth conditions were as described for Fig. 17.
Folate was obtained and assayed as outlined for Fig. 18.

LEGEND:

a. E. coli K-12-AB-301

○—○ 0 ug/ml. sulfadiazine
■--■ 0.5 ug.ml. sulfadiazine
▽--▽ 1.0 ug.ml. sulfadiazine

b. E. coli K-12-AB-301-5

○—○ 0 ug/ml. sulfadiazine
■—■ 250 ug/ml. sulfadiazine
▽--▽ 500 ug/ml. sulfadiazine

c. E. coli K-12-AB-301/222

○—○ 0 ug/ml. sulfadiazine
■—■ 250 ug/ml. sulfadiazine
▽--▽ 500 ug/ml. sulfadiazine

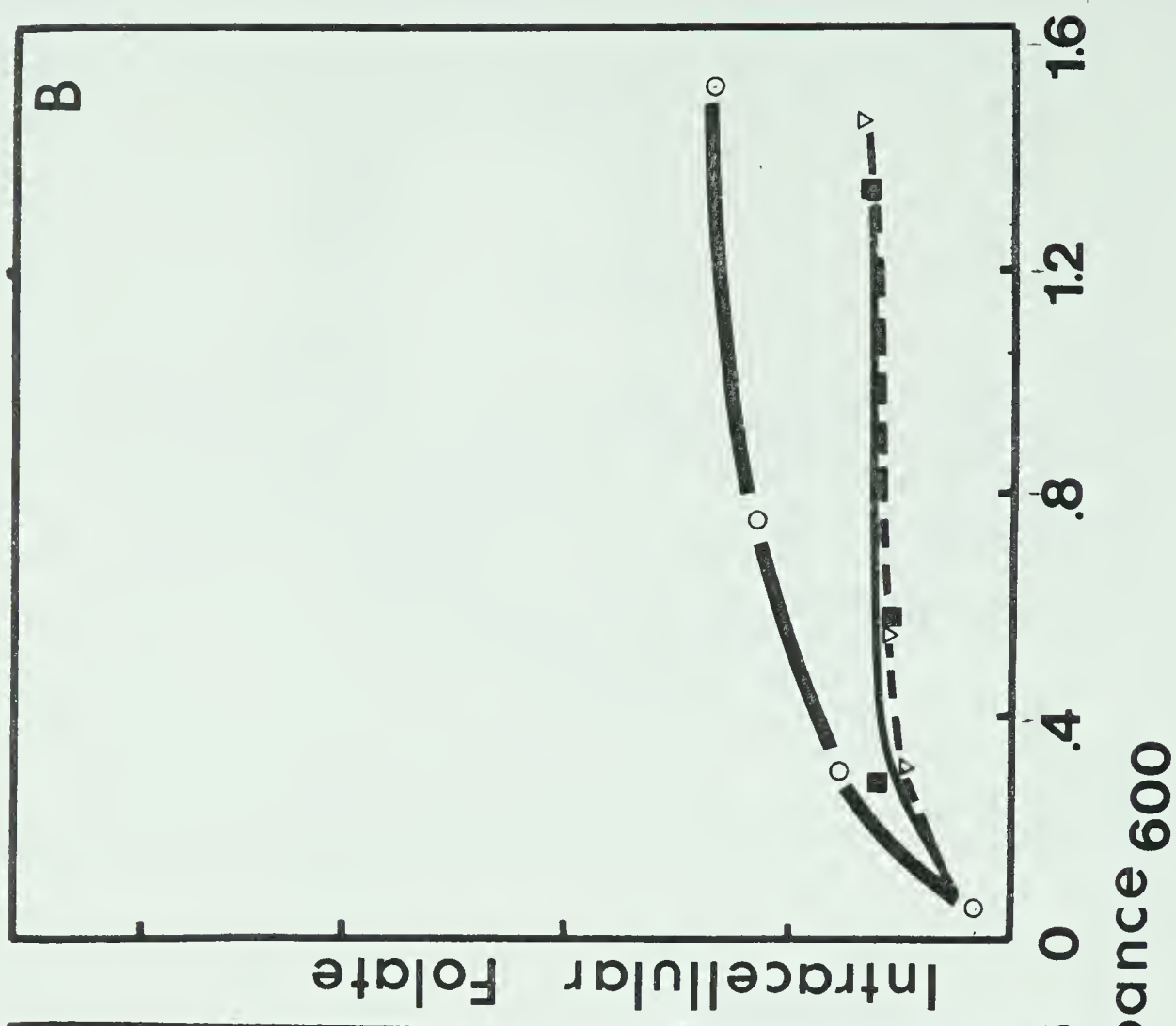
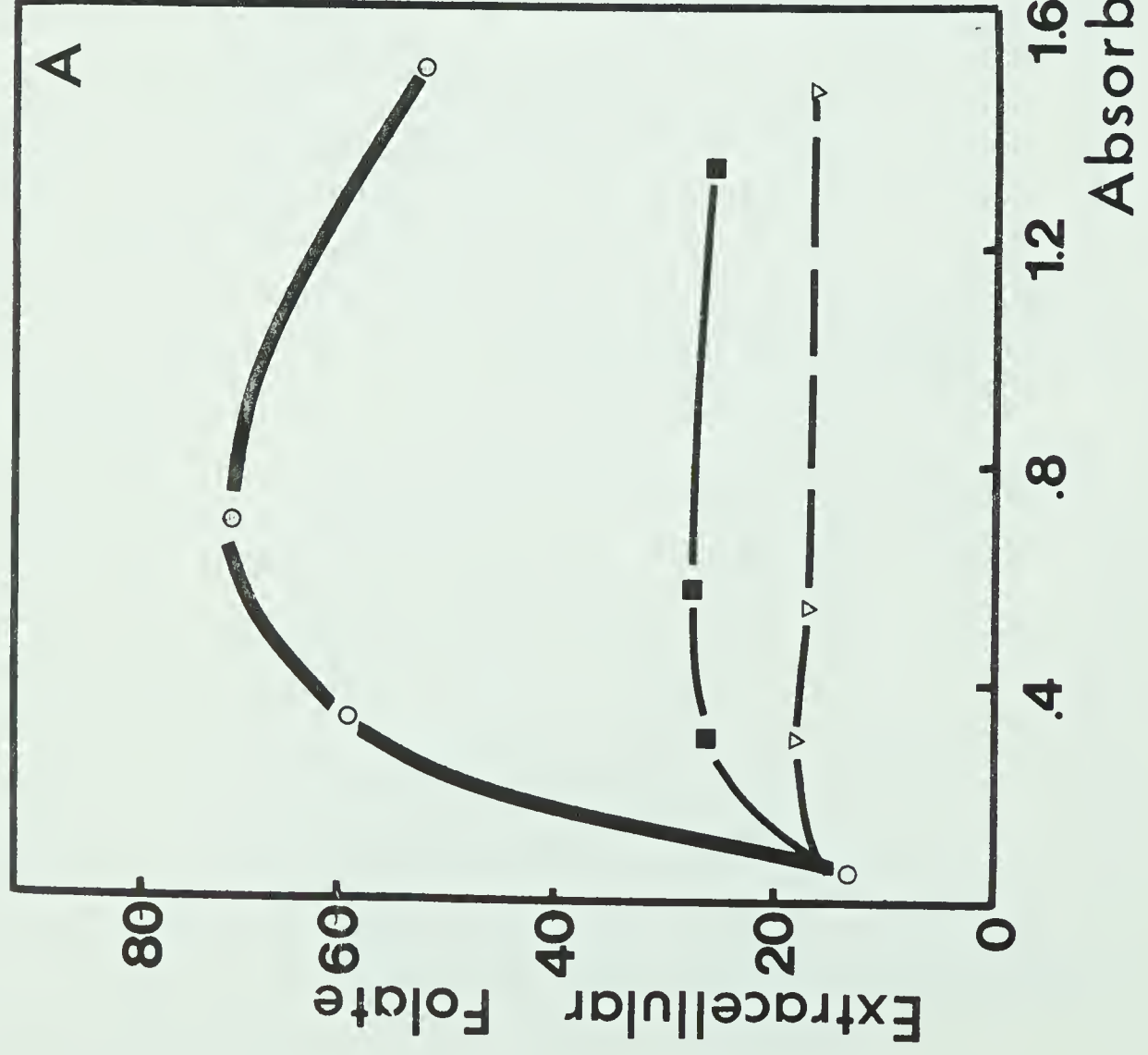


FIGURE 20

Comparison of the production of intracellular and extracellular forms of folate in growing cultures of E. coli K-12-AB-301.

Folates were obtained and analysed as described for Fig. 18.

LEGEND:

○—○ - 0 ug/ml. sulfadiazine

■—■ - 0.5 ug/ml. sulfadiazine

▽—▽ - 1.0 ug/ml. sulfadiazine

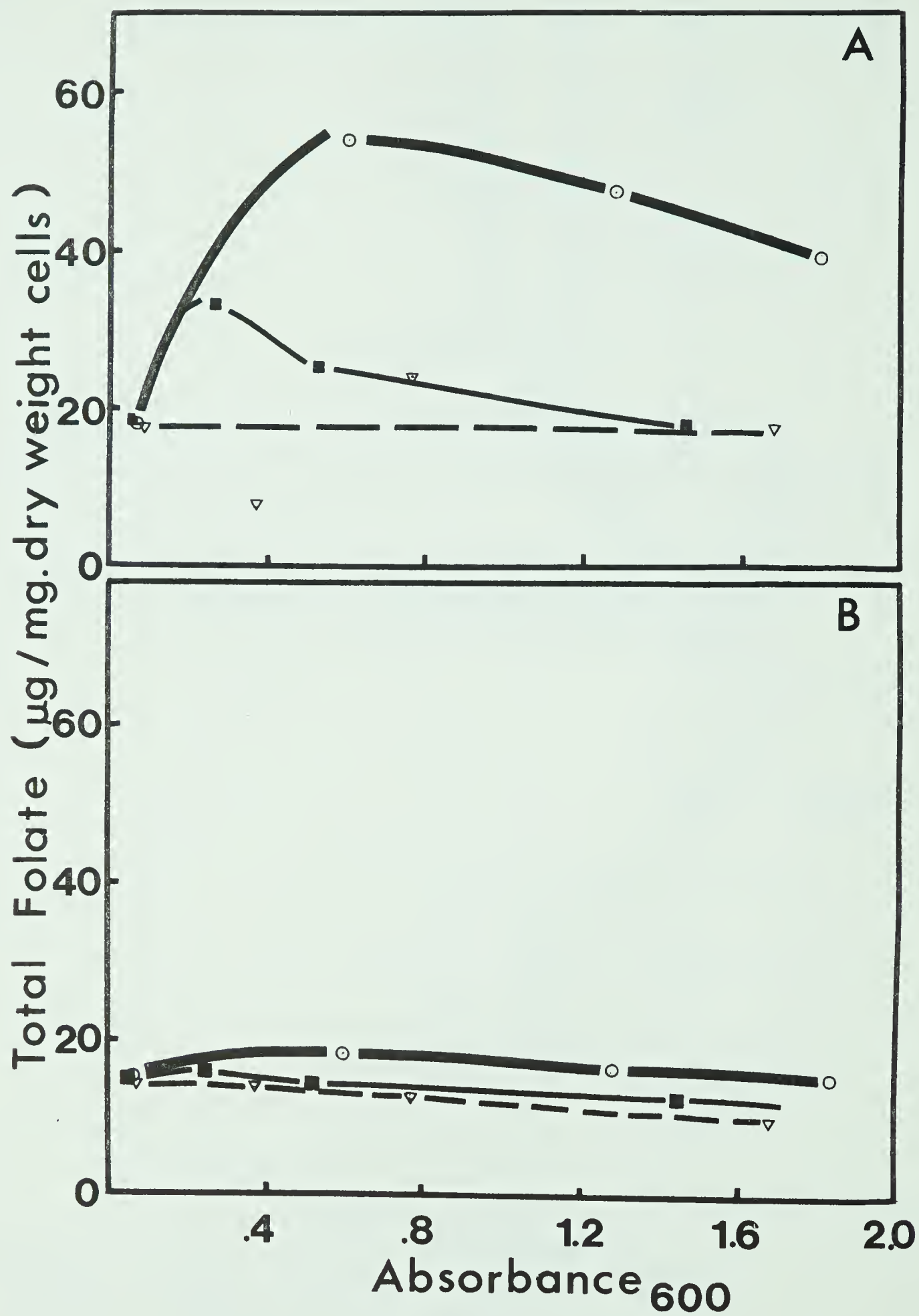


FIGURE 21

Comparison of total folates synthesized by growing cultures of E. coli K-12-AB-301/222 when assayed by L. casei and S. faecalis microbiological assay methods.

Extractions of intracellular folates was carried out by enzymatic hydrolysis; microbiological analysis of folates was done as outlined in Materials and Methods.

LEGEND:

A. L. casei assay system

○-○ - 0 ug/ml. sulfadiazine
■-■ - 250 ug/ml. sulfadiazine
▽-▽ - 500 ug/ml. sulfadiazine

B. S. faecalis assay system

○-○ - 0 ug/ml. sulfadiazine
■-■ - 250 ug/ml. sulfadiazine
▽-▽ - 500 ug/ml. sulfadiazine

p-Aminobenzoic Acid Pools

The 2-fold higher folate level in resistant strains as compared with the sensitive strain was consistent with the finding that there is a 2-fold difference in the specific activities of the sensitive and resistant strains. This may suggest that the mechanism of resistance is also related to pAB pool levels. As shown in Table IX, the pAB levels in both episomal and chromosomal resistant mutants was slightly greater than in the sensitive strain. Extracellular pAB levels were similar in all strains.

Uptake of Sulfonamides

Decreased permeability was considered as a possible mechanism of resistance to sulfonamides. Uptake studies using sulfadiazine - ^{35}S were attempted but no uptake could be demonstrated, even in the sensitive strain. Subsequent uptake studies were done using p-aminobenzoic acid - ^{14}C to see if pAB would be taken up and whether or not sulfonamide would affect the uptake. In both sensitive and resistant strains, ^{14}C - pAB is taken up linearly for 40 to 60 minutes (Fig. 22). In the presence of subinhibitory concentrations of sulfadiazine, pAB uptake was affected in resistant strains but not in the sensitive strain (Fig. 22); however, because of the difference in the concentration of sulfonamide used for studies with sensitive and resistant strains, the results could not be interpreted as a true difference in the pAB transport systems of these two strains.

TABLE IX

ρ -Aminobenzoic Acid Levels in Sensitive and Resistant

Strains of E. coli K-12-AB-301

Strain	ρ AB Concentration		
	Intracellular (ng/mg dry wt.) $\times 10^2$		Extracellular
	Boiled Extract	Washes	Total (ng/ml)
K-12-AB-301	8.5	3.5	13.3
	10.9	3.5	
K-12-AB-301-5	14.9	4.7	21.2
	19.2	3.9	
K-12-AB-301/222	26.2	4.1	29.0
	22.5	5.1	

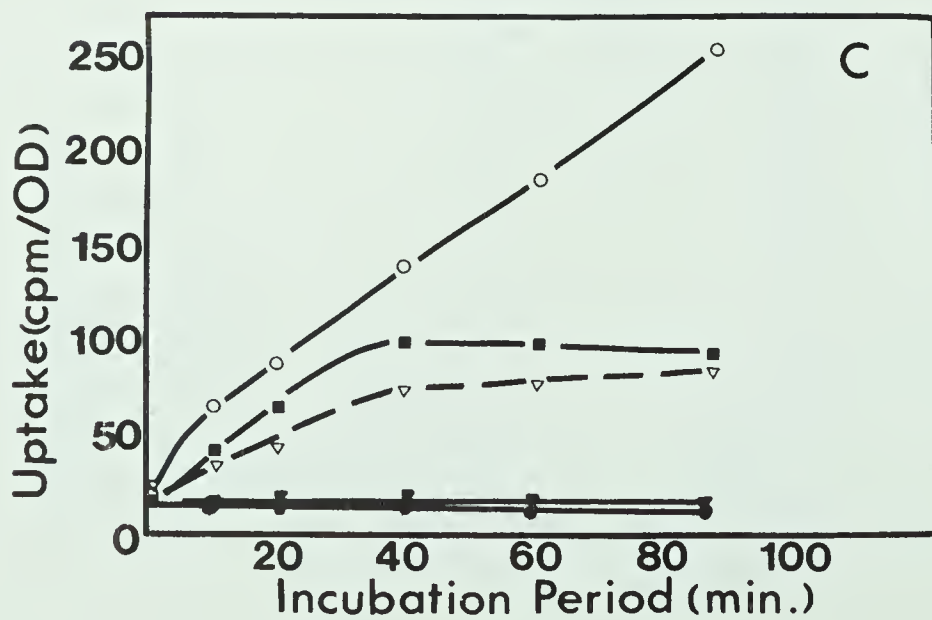
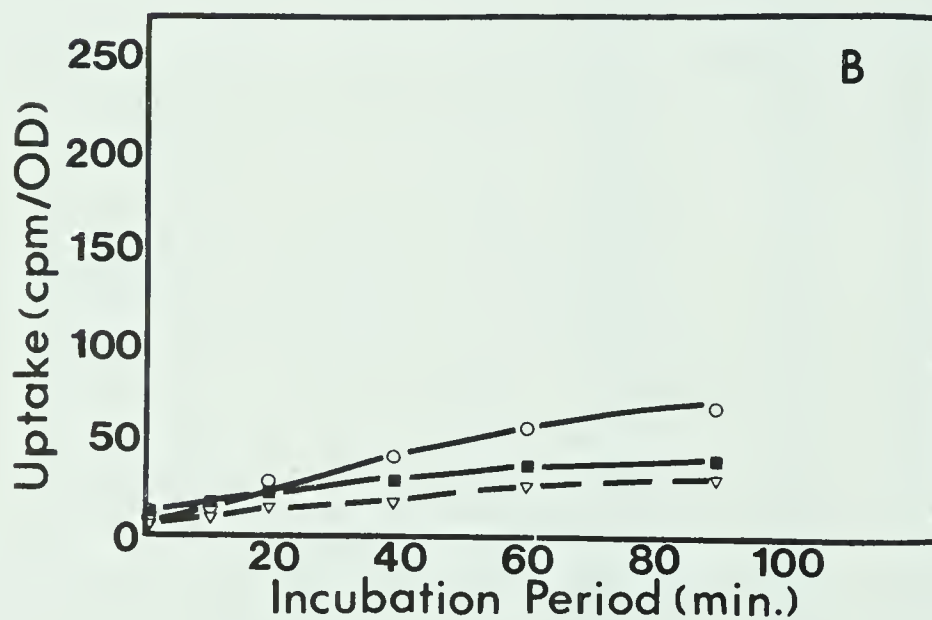
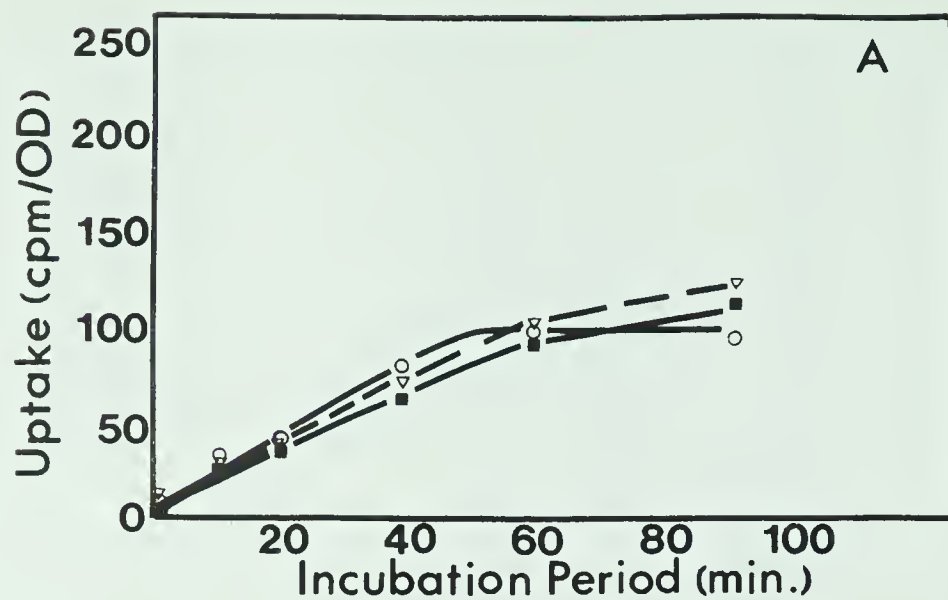


FIGURE 22

Uptake of ρ -aminobenzoic acid - ^{14}C in vivo in sensitive and resistant strains of E. coli K-12-AB-301 in the presence and absence of sulfonamides.

Cells were grown up overnite, washed and resuspended to 500 Klett units in Davis-Mingioli medium. To one (1.0) ml. of uptake menstrum was added 0.01 mM (0.2 uC) of ^{14}C - ρ AB. Growth and uptake were measured in the absence and presence of sub-inhibitory concentrations of sulfadiazine. One (1.0) ml. of cell suspension was added to the uptake menstrum and 100 ul. aliquots removed at 0, 10, 20, 40, 60 and 90 min. Washing and counting procedures are outlined in Materials and Methods.

LEGEND:

A. E. coli K-12-AB-301

○ - ○ - 0 ug/ml. sulfadiazine

■ - ■ - 0.5 ug/ml. sulfadiazine

▽ - - ▽ - 1.0 ug/ml. sulfadiazine

B. E. coli K-12-AB-301-5

○-○- 0 ug/ml. sulfadiazine

■-■- 250 ug/ml. sulfadiazine

▽-▽- 500 ug/ml. sulfadiazine

▼-▼- 0°C control^{*}

●-●- "Boiled cells" control^{**}

C. E. coli K-12-AB-301/222

Symbols as for B.

^{*}0°C control - uptake sample was kept on ice during uptake period.

^{**}"Boiled cells" control - cell suspension was placed in a boiling water bath for 10 min., cooled quickly and used in the uptake experiment at 37°C.

DISCUSSION

Synthesis of Folates in Cell-Free
Extracts of E. coli

In the synthesis of folate in cell-free extracts of E. coli, the requirements for the reaction were similar to those of the system described by Brown et al (1961) and Shiota et al (1964); all required either pAB or pABG as a substrate in addition to a reduced form of pteridine. The systems also required magnesium ion and ATP, shown to be essential for the pyrophosphorylation of reduced pteridine prior to the condensation reaction (Shiota et al, 1964)

Although Brown's initial work on the E. coli system was done at pH 7.0, his later studies (1963, 1964, 1969) were carried out in a pH range of 8.0 - 8.6; the optimum pH of the reaction mixtures used in this study was 8.0. Richey and Brown (1969) suggested that anaerobic conditions for the enzyme assay were not necessary. Similar conclusions were made by Ortiz (1970) and have been confirmed in the present investigation (Fig. 14). It appeared that the concentration of 2- mercaptoethanol (67 mM) which was added to the reaction mixture was sufficient to keep the components of the mixture in the reduced state during the assay period.

All folate enzyme assay systems reported in the literature have been employed with an incubation temperature of 37°C. Although enzyme activity was highest at this

temperature (Fig. 12), it was demonstrated that enzyme activity was not linear with time at 37°C (Fig. 13). Despite a lower enzyme activity, an incubation temperature of 30°C showed linearity of activity with time and was therefore used in the present investigation.

Before any attempt was made to investigate the mechanism of drug resistance, the products of folate biosynthesis were examined. The results of combined paper chromatography and bioautography differed in some respect from those of Brown et al. (1961). When p-aminobenzoic acid was used as substrate, the reaction product appeared to be pteroate (Fig. 7); however, no distinction could be made between pteroate and its reduced forms because of the lack of a dihydropterolate standard and the inability to separate these compounds in the solvent system used. According to the findings of Brown et al. (1961), when glutamate is added to the system, one would expect to find pterolate, folate and/or reduced forms of these compounds. Failure to find folate or its reduced forms may indicate that the activity of the enzyme which catalyzes the condensation reaction of dihydropterolate with glutamate is very low.

When pAB and glutamate were replaced by pABG, as substrate, 3 spots analogous to pterolate, folate and tetrahydrofolate spots were observed. Since no pAB was found as contaminant in pABG samples, there is a possibility that pABG may have been hydrolyzed incompletely to pAB and glutamate. This would account for the presence of a pterolate spot. The apparent tetrahydrofolate spot that was only faintly detectable

might indicate that dihydrofolate is an intermediate in the reaction where ρ ABG is the actual substrate. Lack of a dihydrofolate standard made this impossible to confirm. Ortiz and Hotchkiss (1963) did find both pteroate and folate when they added ρ ABG as substrate in the Pneumococcus system; Brown (1961), on the other hand, found only folate. No conclusive evidence for the folate biosynthetic pathway using ρ ABG as substrate has yet been presented in the literature.

Mechanism of Sulfonamide Resistance in E. coli

Although the present investigation failed to reveal any clear-cut evidence for the mechanism of resistance operating in episomal and chromosomal sulfonamide-resistant mutants of E. coli, several interesting observations have been made.

Examination of the growth patterns of sensitive and resistant strains in the presence and absence of sulfonamides revealed a striking difference between the strains. Both episomal and chromosomal mutants displayed up to 500 times more resistance to sulfonamides than did their sensitive parent strains in "50% limiting sulfonamide level" studies using a defined medium. However the folate-synthesizing enzymes of both sensitive and resistant strains were equally sensitive to sulfonamides. A lower concentration of sulfonamide appeared to be required to limit growth to the 50% level than was required to limit folate synthesis in the cell-free system.

According to Pato and Brown (1963) the difference in sulfonamide sensitivity between sensitive and resistant strains

was only 10-fold. Secondly, a higher concentration of sulfonamide was required to inhibit growth to the 50% level than was required to restrict folate synthesis. The apparent differences between the present study and that of Pato and Brown (1963) might be explained by differences in the procedures used for isolation of mutants; multi-step selection methods were used by Pato and Brown whereas chemical mutagenesis was used for this investigation.

The differences in the "50% limiting sulfonamide level" studies may be attributed to the criteria used for measurement of inhibition. Pato and Brown (1963) added ρ AB to both growth and cell-free systems whereas in the present study, ρ AB was added only to the cell-free system. In both cases, there is a possibility that sulfonamides may be limited in their action on resistant strains by permeability barriers present in the cells. The differences in "50% limiting sulfonamide levels" in growth studies and cell-free systems when different sulfonamides were tested reflects patterns similar to those found by Brown (1962) and Pato and Brown (1963).

Since the same degree of sensitivity to sulfonamides in cell-free systems of sensitive and resistant strains was apparent, attempts were made to determine K_m and K_i values. In the E. coli system, the K_m values for ρ AB were similar; K_i studies produced no results due to inconsistencies of the assay technique. This may be related to the degree of purity of the enzyme fraction used.

Wolf and Hotchkiss (1963), Ortiz and Hotchkiss (1966) and Ortiz (1970) have examined the folate enzyme system in sensitive and resistant strains of Pneumococcus. Using kinetic studies, they have concluded that enzymes from drug-resistant strains have a reduced binding capacity for sulfonamides.

Correlation of folate synthesis in vivo with the levels of folate-synthesizing enzymes present during growth has suggested a possible mechanism of resistance. At the mid-logarithmic phase of growth, specific enzyme activity and in vivo folate synthesis were both approximately 2-fold higher in resistant strains than in the sensitive strain. In the presence of subinhibitory concentrations of sulfonamide, folate synthesis was reduced to one-half that in the absence of sulfonamide in both sensitive and resistant strains; in other words, the presence of sulfonamide reduces folate synthesis in the resistant strain to the same level of synthesis as in the sensitive strain in the absence of sulfonamide. Because the resistant strain may actually grow in the presence of higher concentrations of sulfonamides, perhaps it is synthesizing more folate than required; hence, when sulfonamide restricts folate synthesis, there is still sufficient folate for the resistant strain to perform its normal metabolic functions. Reduction of folate synthesis in the sensitive strain may inhibit growth as a result of "folate starvation." White and Woods (1965 a) considered folate levels as a possible mechanism of chromosomal resistance in a Staphylococcus aureus system; however, they were unable to establish any general pattern in

the several chromosomal mutants which they investigated Yakota (1964) was able to demonstrate 4-5 times higher intracellular folate levels in chromosomally resistant strains of E. coli than in either the sensitive or episomally-resistant strain. In the latter case, this gave support to the hypothesis of different resistance mechanisms existing in episomal and chromosomal sulfonamide-resistant organisms. No such evidence was forthcoming in the present investigation.

Another factor which must also be considered as a possible reason for resistance is the level of p-aminobenzoic acid pools. Since pAB is directly involved in substrate competition with sulfonamide, (Brown et al, 1961) one might expect to find increased p-aminobenzoic acid levels in resistant strains that could overcome this competitive inhibition. White and Woods (1965 a) were able to find that a few chromosomally-resistant strains had 5 to 150-fold higher pAB levels than did their sensitive parent strains. This increase was reflected also in increased folate levels in the same organisms. In the present study, the 2-fold difference found between pAB pool levels in sensitive and resistant strains may or may not be significant. Certainly it is reinforced by folate levels, but the difference in pAB levels, is actually very small.

Because other workers have indicated the possibility of sulfonamide resistance being due to permeability barriers in episomally-resistant mutants, and because there appeared to be no definitive conclusion regarding the resistance mechanism

at this point, uptake studies using ^{35}S -sulfadiazine were undertaken. White and Woods (1965 b) were unable to demonstrate any difference in uptake between sensitive and chromosomally resistant strains. On the other hand, Yokota (1964) demonstrated increased uptake of ^{35}S -sulfathiazole in chromosomal mutants as compared to sensitive strains, but showed significantly decreased uptake by episomally-resistant organisms. The present investigation failed to demonstrate any ^{35}S uptake at all. This in turn may be attributed to problems in setting up a working uptake assay system. Because of this problem, the ^{14}C - ρAB uptake system was investigated in the presence and absence of sulfonamides. If ρAB and sulfonamide were being incorporated via the same transport system, one would expect to find ρAB uptake decreased in the presence of sulfonamides. However, because subinhibitory concentrations of sulfonamide were used in the study, it was impossible to discern whether or not the actual ρAB transport system is affected differently in sensitive and resistant strains.

In the episomal and chromosomal mutants, ρAB uptake was definitely affected by the presence of sulfonamide, suggesting that in these systems ρAB and sulfonamide may enter the cell via the same transport system. In the present study, however, ρAB was not routinely added to the growth medium or to the uptake medium; hence one would not expect any differences in the cells due to competition between ρAB and sulfonamide.

From the foregoing discussion, it may be seen that no definitive conclusions may be reached regarding the mechanism

of sulfonamide resistance in either episomal or chromosomal sulfonamide-resistant organisms. The correlation of folate enzyme activities with folate levels in vivo and in vitro suggests evidence for excess synthesis of folate in both episomal and chromosomal resistant strains as a possible mechanism of resistance; however, due to lack of experimental data, one may not rule out permeability barriers or kinetic differences in folate-synthesizing enzymes as mechanisms of resistance. As suggested by Kabins et al (1971) several of these possible mechanisms may contribute to some extent to the overall resistance of the organisms.

Two areas on which further study remain to be done are uptake measurements and kinetic analysis. Uptake studies with ρ AB should be performed in the presence and absence of inhibitory sulfonamide concentrations and the uptake assay system for sulfonamides must be perfected. Since growth is affected within 2-3 hours after addition of sulfonamides, sulfonamide must be taken up by the cells in a similar or shorter period. Attempts should be made to demonstrate uptake within this period, not over an extended period as has been reported by some workers (Unowsky and Krainski, Bact. Proc. p. 54, 1971).

Kinetic analyses, if pursued, should be considered in more purified systems that were used in this study. Since a multi-enzyme system is involved in folate biosynthesis, it may be necessary to consider separate reaction steps in order to obtain meaningful kinetic data.

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